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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : G01N 33/543, 33/74	A1	(11) International Publication Number: WO 99/46597 (43) International Publication Date: 16 September 1999 (16.09.99)
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(54) Title: ASSAY OF IGFBP COMPLEX (57) Abstract A method of assaying the growth hormone status of an individual by immuno-assay for the 150KDa Insulin-like Growth Factor ternary complex (IGF/IGFBP-3/ALS). Alternatively, a binary complex may be assayed. The method involves capturing the IGFBP complex with a first antibody coupled to a solid phase and detecting the complex with a second antibody coupled to a label. A set of monoclonal and polyclonal antibodies useful for the assay is also provided.		

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ASSAY OF IGFBP COMPLEX

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

5 This invention relates to methods of determining the growth hormone status of an individual. This is performed by measuring the circulating Insulin-like Growth Factor binary or ternary complex (150 KDa complex containing IGF, IGFBP and ALS). The complex is first captured with a monoclonal antibody that is coupled to a solid support and called a "capture-antibody" herein. One of the members of the complex is detected with a monoclonal antibody
10 called a "detection-antibody" herein.

Ideally, the capture- and detection-antibodies do not interfere with each other or with complex formation. For this reason, we have prepared a series of monoclonal antibodies and mapped their epitopes. The invention also pertains to said monoclonal antibodies and kits containing the same.

2. BACKGROUND

15 The super family of insulin-like growth factors (IGFs) includes IGF-I and-II, at least six high affinity IGF binding proteins (IGFBP-1 to IGFBP-6), an acid-labile protein subunit (ALS), and various cell surface receptors, proteases and antagonists. The IGF superfamily is intimately
20 involved in the regulation of cellular growth and metabolism (1-4). IGFs are produced by multiple normal and malignant tissues and are present in blood and other biological fluids in close association with IGFBPs which specifically bind and modulate their bioactivities (4-9). IGF-I, a 7.5 KDa peptide, is the major mediator of the *in vivo* mitogenic and metabolic effects of growth hormone (GH) and, as with IGF-II, its actions are mediated by both endocrine and autocrine-
25 paracrine mechanisms (1-4).

Most of the IGF circulates with an approximately 150 KDa ternary protein complex consisting of IGFBP-3 and ALS (4-9). Binding of ALS to IGFBP-3 depends on IGFBP-3 occupancy by IGF-I or IGF-II and under normal conditions, nearly all of the circulating IGFs (>95%) are found in the ternary complex (4-12). IGFBP-3 is normally the major carrier of IGF
30 in serum and its association appears to stabilize daily IGF levels (half-life of ~ 12-15 hrs) and limit IGF access to extracellular compartments (4-9). It is presumed that most IGFs are found in the

ternary complex because ALS is present at significantly higher molar excess than IGFBP-3 or the IGF peptides (13, 14). Smaller proportions of circulating IGFs are associated with other IGF binding proteins and less than 1% has been estimated to exist in an unbound or "free" form.

While the precise biological relevance of the ternary complex has not been clearly defined, circulating levels of its constituents, and thus formation of the ternary complex, is highly GH-dependent (4-9). Serum IGF-I, IGFBP-3 and ALS are low in GH deficiency and are elevated in acromegalic subjects (4-9, 13, 15). Post-translational modifications of IGFBPs, including proteolysis (16, 17) and phosphorylation (18, 19), are apparently involved in modulating bioavailability of the IGF peptides.

The clinical assessment of GH status has been controversial, primarily due to the episodic nature of GH secretion, its relatively short circulating half-life and considerable variability in GH measurement by different methods (20, 21). Currently, clinical evaluation of GH sufficiency may involve multiple venous blood sampling for determination of GH secretion in response to a number of physiological or pharmacological stimuli. Because of the reported limitations of provocative GH testing, which include arbitrary definition of diagnostic cut-off levels, and potential health risk and cost, alternative screening procedures have been sought (20, 21).

As blood IGF-I, IGFBP-3 and ALS levels are highly dependent on GH secretion (4-9, 13, 15), appropriate determination of their serum levels have been endorsed as the most effective means in the evaluation of GH-IGF axis status, particularly in children with short stature (21-23). Determinations of IGF-I and ALS may be also valuable in the diagnosis of adult GH deficiency and monitoring of therapeutic response to GH replacement therapy (24, 25).

The high levels of association with IGFBPs has been a major concern in routine immunoassay of serum IGF-I because IGFBPs may mask reactive epitopes or compete with antibodies for tracer binding. Reliable determination of IGF-I in serum requires dissociation and removal of IGFBPs before analysis (26). Present strategies are based on serum acidification to irreversibly denature ALS and disrupt the ternary complex, followed by procedures to remove most or all of the IGFBPs. Size exclusion chromatography in acid is considered the "gold standard" method for the latter step (27), but is not practical for efficient high-volume sample processing.

The most commonly used alternative is acid-ethanol precipitation (28). However, the method may leave substantial residual amounts of IGFBPs (19, 29-32). We recently confirmed

that IGFBPs, in particular low molecular weight IGFBPs such as IGFBP-1, may be retained in significant quantities after acid-ethanol precipitation (19). The residual amounts of IGFBPs could compete with the detection-antibody, particularly in competitive RIAs (29) where the amount of antibody and labelled IGF-I is small relative to the concentration of analyte and residual IGFBPs. This phenomenon may account for the well known propensity of IGF-I RIAs to give false estimates of IGF-I levels in samples with high levels of endogenous IGFBPs (27, 29, 30, 32).

Various means have been devised to minimize the interference of residual IGFBPs, including addition of IGF-II to the assay mixture, acid-ethanol cryoprecipitation, use of analog tracers with low affinity for IGFs or acid-Bio-Spin chromatography (29, 30, 32, 33). However, these methods further add to the complexity of the assay and may not completely resolve the IGFBP-interference problem.

The clinical assessment of IGF-I, IGFBP-3 and ALS is further complicated by the differential effects of age, pubertal stage of development and nutritional factors on their circulating levels (21, 34, 35). Accordingly, current recommendations suggest use of IGF-I, IGFBP-3 and ALS in younger age group, and IGF-I and ALS in adult subjects (21-26, 36). As nearly all of serum IGF-I, IGFBP-3 (4-12) and as much as 50% of ALS (13, 15) are present in the high molecular weight IGFBP-3 ternary protein complex, direct determination of the complex may represent an ideal and potentially superior screening alternative. However, until now, no one has presented a reliable, accurate and simple means of quantifying the level of IGFBP complex.

SUMMARY OF THE INVENTION

ABBREVIATIONS AND DEFINITIONS

ALS - Acid Labile Subunit - A protein found in the 150 KDa ternary complex where most of the circulating IGF is found. ALS is sensitive to inactivation by acid.

Antibodies - The antibodies described herein include ten mouse monoclonal antibodies against recombinant human IGFBP-3 called B1 to B10; a polyclonal antibody against recombinant human IGFBP-3 called pB11, two mouse monoclonal antibodies to the C and N terminals of recombinant human IGF-I called I-1 and I-2, respectively; a polyclonal antibody to recombinant human IGF-II called pI-3, and two polyclonal antibodies to N and C terminal peptide fragments of human ALS called pA-1 and pA-2, respectively. Additional antibodies may be generated by

methods well known in the art and evaluated as described herein.

Binary complex - A two part complex of IGFBP and ALS or of IGFBP and IGF.

Body fluid - Any biological fluid, including but not limited to the following: serum, plasma, lymph fluid, synovial fluid, follicular fluid, seminal fluid, amniotic fluid, milk, mammary fluid, whole blood, urine, spinal fluid, saliva, sputum, tears, perspiration, mucus tissue culture medium, tissue extracts and cellular extracts.

BSA - Bovine serum albumin.

Capture-antibody - An antibody that can be used to capture the IGFBP complex.

Capture-antibody coupled to a solid support - The coupling of the antibody to the solid support may be any of the well known coupling means, including covalent, non-covalent, magnetic means and the like. Commonly used cross-linking agents for attaching the capture-antibody to the solid phase substrate include, for example, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, homobifunctional imidoesters, and bifunctional maleimides. Derivatizing agents, such as methyl-3-[(p-azidophenyl) dithio]propioimide, yield photoactivatable intermediates capable of forming cross-links in the presence of light. The coupling of antibodies to various solid supports is well known in the art and need not be detailed herein.

Control standard - A sample of IGFBP complex of known concentration.

Detection-antibody - An antibody that can be detected in some manner.

Detection-antibody coupled to a label - A detection-antibody labelled by any means. The detection-antibody may be inherently labelled, for example by an incorporated radioisotope or modified amino acid or by its species specific antigenic determinants. Additionally, the antibody may be labelled by means of a detectable moiety (label) coupled to the antibody.

ELISA 1 - An ELISA based on the capture of IGFBP complex with anti-IGFBP B3 and detection with anti-IGF-I I-1.

ELISA 2 - An ELISA based on the capture of IGFBP complex with anti-IGFBP B2 and detection with anti-IGF-I pA-1.

GH - Growth hormone.

GHRD - Growth hormone receptor defective.

GHD - Growth hormone deficiency.

GH status - The growth hormone status of an individual is reflected in the levels of GH,

IGF, IGFBP, IGFBP complex or ALS as well as by other molecules related to the GH and IGF cascades. The GH status of an individual is known to vary -either up or down- in certain growth-related disease states, such as hypoglycaemia, diabetes, deficient nutritional states, certain cancers such as breast cancer and prostate cancer, immune deficiencies, fetal growth retardation, gigantism, acromegaly, hyperpituitarism, pituitary dwarfism, GH deficiency, GH excess, GH receptor defect, thyromegalia and certain CNS diseases such as Alzheimer. Therefore, many diseased individuals (human or animal) could benefit from the monitoring of growth hormone status. Additionally, there are other applications such as the monitoring of nutritional status, reproductive health, performance of racing and other performance individuals, improved breeding stock, and the like.

HRP - Horse radish peroxidase.

IGF - Insulin-like Growth Factor.

IGFBP - An IGF binding protein, including IGFBP-1 to 6 and the heretofore unsequenced IGFBPs. Preferably, the IGFBP is IGFBP-3 in the context of the assay described herein.

IGFBP-3 - The major circulating IGF binding protein.

IGFBP complex - This term is defined herein to include either the binary complex of IGFBP and ALS or IGF or the ternary complex of IGFBP and ALS and IGF.

Label - Any detectable marker or detectable functionality that does not interfere with the binding of the antibody to its epitope, including but not limited to, a radioisotope, an enzyme, a fluorescent label or other measurable tag as described in U.S. Pat. No. 4,275,149 and U.S. Pat. No. 4,318,980. Numerous labels are known for use in immunoassay, including those that may be detected directly, such as fluorochrome, chemiluminescent and radioactive labels, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. Examples of labels include the radioisotopes ^{32}P , ^{14}C , ^{125}I , ^3H , and ^{131}I , fluorophores such as rare earth metals or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luciferases, luciferin, green fluorescent protein, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, beta-galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as horse radish peroxidase, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

Additionally, the antibody itself may inherently comprise a label, for example where the detection means involves a second antibody directed against antibodies from the species from which the antibody was obtained. The preferred label is horse radish peroxidase or biotin. The number and types of labels are numerous and need not be fully described here.

5 **PSA** - Prostate specific antigen.

RT - Room Temperature.

SD- Standard deviation.

Solid support - A solid phase used for immobilization of the capture-antibody which may be any inert support or carrier that is essentially water insoluble and useful in immunoassays, including supports in the form of surfaces, particles, porous matrices, etc. Examples of commonly used supports include small sheets, Sephadex, polyvinyl chloride, plastic beads, and assay plates or test tubes manufactured from polyethylene, polypropylene, polystyrene, and the like, including 96-well micro-titre plates, as well as particulate materials such as filter paper, agarose, cross-linked dextran, and other polysaccharides. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537 and 4,330,440 are suitably employed for capture-antibody immobilization. The preferred solid phase used herein is a multi-well micro-titre plate that can be used to analyze several samples at one time. The number and types of solid supports are numerous and need not be fully described here.

20 **Ternary complex** - The 150 KDa complex composed of IGF, IGFBP and ALS.

Treatment designed to influence growth hormone status - Includes any medical treatment whose intended effect is to influence the GH or IGF cascades. Treatments may include treatments with such agents as GH, GHBP, IGF, IGFBP, ALS, IGFBP complex, GH receptors, IGF receptors, antibodies or inhibitors of any of the preceding, receptor antagonists for GH or IGF, or any drug that acts to modulate the growth hormone status of an individual, including tamoxifen, somatostatin, somatostatin analogues, GH antagonists, IGF antagonist, IGFBP stimulators, retinoids, TGF-beta, and vitamin D analogues, and the like. Individuals include both human and animals, such as pigs, cattle, sheep, goats, horses, poultry, cats, dogs, fish, etc.

We have recently described non-competitive ELISAs for the determination of IGF-I (37), IGF-II (38), IGFBP-1 (19), IGFBP-3 (39) and ALS (15) in serum and other physiological fluids. Clinical assessment of GH-related disorders and monitoring of GH therapeutic regimes

increasingly involve biochemical determinations of circulating IGF-I, IGFBP-3 and ALS levels (21-26, 36). As nearly all of blood IGF-I and IGFBP-3 (4-12) and as much as 50 percent of ALS (13, 15) are normally confined within the GH-dependent 150 KD ternary complex, direct determination of the complex may have significant advantages. We hereby report the development of highly specific and rapid ELISAs for the direct measurement of the high molecular weight IGF-binding protein complexes.

Generally speaking, the method for quantitative measurement of IGFBP complex involves capture of the complex with a first capture-antibody, washing away all unbound components, and detecting the remaining complex with a second detection-antibody. The possible immunoassay designs are based on numerous "capture and detection-antibody" combinations, and may involve combinations of (a) anti-IGFBP-3 antibodies, (b) anti-IGF-I antibodies, (c) anti-ALS antibodies, and (d) anti-complex antibodies, provided that each antibody pair (or triplet) reacts with separate epitopes and does not interfere with complex formation.

More specifically, we have exemplified the invention with an anti-IGFBP-3 capture-antibody and detection by an antibody directed against either ALS or IGF. Our approach eliminates the need for complex sample pre-treatment procedures, allows combined determination of the IGFBP complex components in a single assay, and potentially provides better clinical information.

Depending on the assay design, the ternary as well as any binary complex may be quantified, while contributions by fragments and less tightly bound or dissociable variants are eliminated.

Two specific capture-detection-antibody pairs are exemplified below and called ELISA-1 and ELISA-2. ELISA-1 involves an IGFBP-3/IGF-I antibody combination and is specific for both IGFBP-3/IGF-I/ALS ternary complexes and any binary IGFBP-3/IGF-I complex that could be potentially present. The specificity of the assay for such binary complexes was established by analysing samples before and after acid-neutralization, which is known to functionally inactivate ALS. ELISA-1 measured similar IGFBP-3 complex levels in untreated and acid-treated samples.

ELISA-2 involves an IGFBP-3/ALS antibody combination and quantifies both IGF-I as well as IGF-II-based ternary complexes and binary IGFBP-3/ALS complex. As expected, ELISA-2 did not detect any immuno-reactivity in acid-treated samples.

It is also possible to modify the assay to detect other entities. For example, it is possible

to detect total IGFBP (complexed and free) with a variation of the ELISA 1 and 2. First, all IGFBP is captured with an anti-IGFBP antibody. Next, the captured IGFBP is saturated with exogenous ligand (either IGF and/or ALS depending on which detection-antibody is employed) before detection with the detection-antibody. In this assay design, both complexed IGFBP and free IGFBP will be captured by the capture-antibody, driven to complete association with the exogenous ligand, and detected by the anti-IGF or anti-ALS detection-antibody.

Further, although we have exemplified assays wherein the capture-antibody is an anti-IGFBP antibody, it is also possible to use anti-IGF, anti-ALS or an anti-complex antibody as the capture-antibody. It is apparent that various assay designs may be optimized by one of skill in the art as described herein.

The invention includes a kit for the assay of an IGFBP-complex. The kit contains a capture-antibody and a detection-antibody, provided that the capture- and detection-antibodies do not bind to overlapping epitopes and do not interfere with said IGFBP complex formation. The capture-antibody may be bound to a solid support and the detection-antibody is generally labelled in some manner.

The capture- and detection-antibody combinations may be selected from the group consisting of B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, pB11, I-1, I-2, pI-3, pA-1 and pA2. However, preferably, the antibody pair is B3 and I-1. In an additional preferred embodiment, the antibody pair is B2 and pA-1.

The kit may contain other useful reagents, such as a label detection means, a binding buffer, a wash buffer, a detection buffer and a control standard, and the like.

The invention also embodies a method of detecting IGFBP complex in a body fluid. The method consists of a) contacting a solid phase coupled to a capture-antibody with a body fluid to capture an IGFBP complex; b) washing the solid phase with a first wash buffer; c) contacting the solid phase with a detection-antibody coupled to a label; d) washing the solid phase with a second wash buffer; and e) detecting said label remaining with said solid phase after said washing step.

This method can advantageously be employed to determine the GH status of an individual, including, but not limited to human, bovine, porcine, equine, canine, feline, or ovine individuals. Other specific applications include, but are not limited to: a) monitoring the effect of treatments designed to influence the GH or IGF status of an individual; b) monitoring the clinical status and/or treatment of individuals with various diseases affected by GH status, including

hypoglycaemia, diabetes, deficient nutritional states, certain cancers such as breast cancer and prostate cancer, immune deficiencies, fetal growth retardation, gigantism, acromegaly, hyperpituitarism, pituitary dwarfism, GH deficiency, GH excess, GH receptor defect, thyromegalia and certain CNS diseases such as, but not limited to Alzheimers; c) determining an individual's susceptibility for growth-related disease states, where susceptibility is characterized by aberrant or elevated GH status; or d) various veterinary applications such as monitoring of nutritional status, reproductive health, performance of racing and other performance indices, detecting improved breeding stock, disease resistance, and the like.

DESCRIPTION OF THE DRAWINGS

Figure 1. Epitope Map of IGFBP-3 Complex.

Four antigenic regions were defined. Overlapping circles indicate no sandwich formation, touching circles indicate interfering sandwich formation, and separate circles indicate independent epitopes.

Figure 2. Inhibition of Antibody Binding to IGFBP-3 by IGF-I and IGF-II.

Binding of anti-IGFBP-3 monoclonal and polyclonal antibodies to IGFBP-3 before and after pre-incubation with 125 -IGF-I or 125 -IGF-II are shown. Anti-PSA antibody was used as a negative control. Values are the mean of three measurements.

Figure 3. IGFBP-3 Complex ELISA Calibration Curve.

Linear-linear plots of typical ELISA-1 and ELISA-2 calibration curves are shown.

Figure 4. Stability of IGFBP-3 Complex Analysed by ELISA-1.

Replicate aliquots of three different samples (S1-S3) stored at various temperature and analysed as indicated. Percent changes from the day 0 value are shown.

Figure 5. Stability of IGFBP-3 Complex Analysed by ELISA-2.

Replicate aliquots of three different samples (S1-S3) stored at various temperature and analysed as indicated. Percent changes from the day 0 value are shown.

Figure 6. Comparison of IGFBP-3 Complex with IGF-I.

EDTA plasma samples from subjects with untreated GHRD (n=11) and age-matched normal subjects (n=16), and serum samples from adults with acromegaly (n=8) or GHD (n=5) were assayed for IGF-I and IGFBP-3 complex by ELISA-1 and ELISA-2. Values are the mean of duplicate measurements.

Figure 7. Comparison of IGFBP-3 Complex with IGF-II.

EDTA plasma samples from subjects with untreated GHRD (n=11) and age-matched normal subjects (n=16), and serum samples from adults with acromegaly (n=8) or GHD (n=5) were assayed for IGF-II and IGFBP-3 complex by ELISA-1 and ELISA-2. Values are the mean of duplicate measurements.

Figure 8. Comparison of IGFBP-3 Complex with IGFBP-3.

EDTA plasma samples from subjects with untreated GHRD (n=11) and age-matched normal subjects (n=16), and serum samples from adults with acromegaly (n=8) or GHD (n=5) were assayed for IGFBP-3 and IGFBP-3 complex by ELISA-1 and ELISA-2. Values are the mean of duplicate measurements.

Figure 9. Comparison of IGFBP-3 Complex with total ALS.

EDTA plasma samples from subjects with untreated GHRD (n=11) and age-matched normal subjects (n=16), and serum samples from adults with acromegaly (n=8) or GHD (n=5) were assayed for total ALS and IGFBP-3 complex by ELISA-1 and ELISA-2. Values are the mean of duplicate measurements.

Figure 10. IGF-axis Components Levels versus Age.

IGF-I, IGF-II, IGFBP-3, ALS and IGFBP-3 complex levels by ELISA-1 and ELISA-2 were measured in EDTA-plasma samples obtained from 16 normal subjects. For each analyte, the individual sample values as a percentage of the corresponding mean values are plotted versus age.

Figure 11. IGF-axis Component Levels as a Percentage of the Mean of Normal Values.

EDTA plasma samples from subjects with untreated GHRD (n=11) and age-matched normal subjects (n=16), and serum samples from adults with acromegaly (n=8) or GHD (n=5) were assayed for IGF-I, IGF-II, IGFBP-3, total ALS and IGFBP-3 complex by ELISA-1 and ELISA-2. The mean concentrations of each analyte measured in each sample group as a percentage of the corresponding mean of the normal values (considered as 100%) are shown.

Figure 12. Distribution Plot of IGF-axis Components and IGFBP-3 Complex

EDTA plasma samples from subjects with untreated GHRD (n=11) and age-matched normal subjects (n=16), and serum samples from adults with acromegaly (n=8) or GHD (n=5) were assayed for IGF-I, IGF-II, IGFBP-3, total ALS and IGFBP-3 complex by ELISA-1 and

ELISA-2. Values represent the mean of duplicate measurements.

DETAILED DESCRIPTION OF THE INVENTION

The capture-detection IGFBP complex assay is exemplified herein in two different formats. However, a variety of antibodies are described and mapped and various combinations of antibody pairs or triplets may be employed in the basic capture-detection assay format. The following examples describe the assays, epitope mapping and antibodies of the invention.

EXAMPLE 1. MATERIALS

A. SAMPLES

EDTA-plasma samples from subjects with untreated GHRD (5 males age 1-32 yr, mean age 17 ± 14.5 , and 6 females age 2.3-65 yr, mean age 29.9 ± 25) and age-matched normal controls (8 males age 1-27 yr, mean age 13.3 ± 10.4 , and 8 females age 2-67 yr, mean age 36.6 ± 23.7) were obtained from Dr. Jaime Guevara-Aguirre, Institute of Endocrinology, Metabolism and Reproduction, Quito, Ecuador. Ecuadorian GHRD patients were selected for this study since they have extremely low levels of IGF-I and IGFBP-3 (20). The sample collection protocol was approved by the Ethics Committee of the Institute of Endocrinology, Metabolism and Reproduction (Quito-Ecuador) in compliance with the laws and regulations of the United States and Ecuador. All subjects and/or guardians signed Spanish versions of the approved informed consent forms. These samples were stored at -70°C until use.

Serum samples from consenting adults with acromegaly ($n=8$) and GH deficiency ($n=5$) were kindly provided by Dr. John Miell, Department of Medicine, Kings College School of Medicine, London, England. Randomly selected fresh serum samples ($n=42$) were obtained from Clinical Laboratories in Canada. These samples were residuals from routine clinical test samples and were from an adult population. Upon collection, blood samples were allowed to clot, separated and, after clinical testing, the residuals stored at 4°C were used for these studies within 48 hours.

B. REAGENTS

Recombinant human IGF-I and IGF-II were obtained from GROPEP, PTY, LTD. (Adelaide, Australia) and recombinant non-glycosylated IGFBP-3, was obtained from CELTRIX

PHARMACEUTICAL, INC. (Santa Clara, CA). Recombinant human IGFBP-2, and IGFBP-4 through IGFBP-6 were purchased from AUSTRAL BIOLOGICALS (San Roman, CA). IGFBP-1 was purified from human amniotic fluid and calibrated against pure recombinant human IGFBP-1 obtained from DSL (Webster, TX). Other materials and chemicals were obtained as described (40, 41). Purified human ALS was obtained from DSL (Webster, TX) as described elsewhere (15). ^{125}I -IGFBP-3 (5×10^6 CPM/mL of 0.1 M NaPO_4 , pH 7.4) was obtained from DSL (Webster, TX). HRP-labelled streptavidin was purchased from AMERSHAM INTERNATIONAL (Buckinghamshire, England).

Horseradish peroxidase (HRP) was obtained from SCRIPPS LABORATORIES (San Diego, CA). Tetramethylbenzidine peroxidase ($\text{TMB}/\text{H}_2\text{O}_2$) substrate system was from KIRKEGAARD AND PERRY LABORATORIES, INC. (Gaithersburg, MD). Sulfosuccinimidyl-6-(biotinamido)hexanoate (NHS-LC-Biotin) was from PIERCE CHEMICAL CO. (Rockford, IL). All other chemical reagents were of highest quality and were obtained from SIGMA CHEMICAL CO. (St. Louis, MO), or AMRESCO, INC. (Solon, OH). Micro-titration strips and frames were products of COSTAR (Cambridge, MA).

C. ASSAY BUFFERS

The IGFBP-3/IGF-I (ELISA-1) and IGFBP-3/ALS (ELISA-2) assays described below are based on similar principles and identical components except for the antibody combination and selection of the assay buffer. The buffers were as follows:

ELISA-1 assay buffer - 0.05 molar Tris-maleate, pH 7.0, 9 g/L NaCl, 5 g/L BSA, 0.001 molar sodium EDTA, 0.5 ml/L Tween 20, 0.1 g/L thimerosal.

ELISA-2 assay buffer - 0.05 molar Tris-maleate, pH 7.0, 0.9 g/L NaCl, 20 g/L BSA, 0.5 g/L bovine gamma globulin, 25 ml/L normal goat serum, 100 mg/L PMSF, 0.5 ml/L Tween 20, 0.1 g/L thimerosal.

Zero standard matrix buffer - 0.05 molar sodium phosphate, pH 7.4, 9 g/L NaCl, 1 g/L BSA, 0.005 molar sodium EDTA, 2.5 ml/L trasylol, 0.1 g/L thimerosal.

Antibody-HRP conjugate concentrate buffer - 0.02 molar sodium phosphate, pH 7.0, 9 g/L NaCl, 1 g/L CaCl_2 , 5 g/L BSA, 0.1 g/L thimerosal.

Stopping solution - 0.2 molar sulphuric acid in deionized water.

Coating Buffer - 50 mM Tris, 0.1% NaAzide, pH 7.8

Wash Buffer - 0.05% Tween 20, 5 mM Tris, pH 7.4

Blocking Buffer - 50 mM Tris, 1% BSA, 0.025 % NaAzide, pH 7.4

D. ANTIBODIES

Methods for preparation of both monoclonal antibodies and polyclonal antibodies are now well established (49). For this invention, the monoclonal antibodies were generated in mice and the polyclonal antibodies were raised in goats. The antibodies could have been raised in various species however, including but not limited to, mouse, rat, rabbit, goat, sheep, donkey, horse, etc. The anti-IGF and anti-IGFBP-3 were raised against unglycosylated recombinant human IGF-I, IGF-II or IGFBP-3 (see Table 1 for antibody names and characteristics). Anti-ALS antibodies were raised against synthetic unique N- and C-terminal regions of human ALS (15). The ALS peptides were conjugated to ovalbumin using glutaraldehyde, mixed with complete Freund's adjuvant and injected into goats (0.1 mg/injection) using a monthly boost and bleed schedule. The ALS peptides were prepared using a Model 430A APPLIED BIOSYSTEMS (Foster City, CA) peptide synthesizer (SYNPEP CORP, Dublin, CA) and purified by high-performance reverse phase liquid chromatography (HPLC). IGF-I, IGF-II and IGFBP-3 antigens were injected as above without modification.

Immunizing Agent	Antibody Name	Characteristics
recombinant unglycosylated IGFBP-3	B1 to B10	ten anti-IGFBP-3 monoclonal antibodies
recombinant unglycosylated IGFBP-3	pB11	anti-IGFBP-3 polyclonal antibody
recombinant unglycosylated human IGF-I	I-1	anti-C-terminal-IGF-I monoclonal antibody
recombinant unglycosylated human IGF-I,	I-2	anti-N-terminal-IGF-I monoclonal antibody
recombinant unglycosylated IGF-II	pI-3	anti-IGF-II polyclonal antibody
synthetic unique N-terminal (ALS ₁₋₃₄) region of human ALS	pA-1	anti-N-terminal-ALS-peptide polyclonal antibody
synthetic unique C-terminal (ALS ₅₅₁₋₅₇₈) region of human ALS	pA-2	anti-C-terminal-ALS-peptide polyclonal antibody

Table 1. Immunogens, Antibody names and Characteristics

For the development of monoclonal antibodies, splenocytes from appropriately immunized BALB/c mice were fused with myeloma cells by the polyethylene glycol method. The viable hybridomas were selected, screened and propagated. The supernatants from selected clones were screened against the corresponding analyte by ELISA technique. The positive hybridomas were cloned by limiting dilution and clones secreting specific antibodies were used for ascites production. All monoclonal antibodies were affinity purified on protein A columns and appropriately screened for specificity using well established western-immunoblot methods (50).

Polyclonal antibodies were first purified by affinity chromatography over gel columns containing the immobilized corresponding immunogen and the antibody fractions further purified by chromatography over Protein-A columns. As outlined below, the specificity of the antibodies was further substantiated by immunoassay cross-reactivity studies. All antibodies were produced, characterized and purified by DSL, INC. (Webster, TX). The anti-IGF-I, anti-IGF-II, anti-ALS and the anti-IGFBP-3 polyclonal antibodies are highly specific for the corresponding analyte (15, 37, 38, 39) and have been employed in corresponding ELISAs manufactured by DSL INC., (Webster, TX). The anti-IGFBP-3 monoclonal antibodies are also highly specific for IGFBP-3 and are currently under evaluation for development of additional novel IGFBP-3 ELISAs.

EXAMPLE 2. METHODS: PREPARATIVE ASSAY PROCEDURES

Antibody coating to micro-titre wells was performed at a concentration of 2.5-30 mg/L, unless otherwise indicated, using previously published methods (40). In brief, 0.1-0.2 mL of the antibody solution (5-10 mg/L) was added to each micro-titre well and allowed to incubate overnight at room temperature. The wells were then washed once with the wash solution and 0.2 mL/well of the blocking solution was added and allowed to incubate for 1 hour as above. The wells were washed once prior to use, or stored for up to 1 week in the blocking buffer at 4°C.

IGFBP-3 coating to micro-titre wells was performed as described above, except that IGFBP-3 was coated at a concentration of 0.25-2 mg/L.

Antibody coupling to HRP was performed as previously described (41). The coupling reaction involved initial activation of the enzyme with sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) and its subsequent conjugation to 2-iminothiolane activated antibody. The stock HRP-conjugated antibody solution was stored at 4°C in the dark and appropriately diluted (at least 1000-fold) in the appropriate assay buffer prior to use.

Antibody coupling to biotin was performed as previously described (42). Biotinylation was performed at about a 150-fold molar excess of NHS-LC-Biotin added to 0.5 mg/mL of the antibody solution. The unconjugated biotin was removed by dialysis at 4°C for 24 hours against several changes of 0.1 molar sodium bicarbonate, pH 8.3, containing 9 g/L of NaCl and 0.25 g/L of sodium azide. The stock antibody-biotin conjugate solution stored at 4°C and appropriately diluted in 0.05 molar NaPO₄, pH 7.2, containing 9 g/L of NaCl, 2 g/L of BSA and 1.0 mL/L of the antibacterial/antifungal preservative Proclin 300 (SIGMA) prior to use.

A pool of fresh serum samples was assigned 100 arbitrary units per litre (AU/L) of IGF-binding protein complex (IGFBP-3 complex) and used for ELISA-1 and ELISA-2 standardization. Standards were prepared by appropriately diluting the serum pool in the zero standard matrix buffer to give reference standard values of 0.78, 1.56, 6.25, 25 and 50 AU/L of IGFBP-3/IGF-I complex, and 3.13, 6.25, 12.5, 25, and 50 AU/L of IGFBP-3/ALS complex for use in ELISA-1 and ELISA-2, respectively. The standards were stable for up to 24 hours at 4°C and greater than 2 months at -20°C or lower. The quality control samples used were also appropriately diluted serum pools. The nominal concentrations of the control samples were established by analysing them in IGFBP-3 complex ELISA-1 and ELISA-2.

EXAMPLE 3. METHODS: IGFBP-3 EPIOTOPE MAPPING

A. SIMULTANEOUS BINDING OF ANTI-IGFBP ANTIBODIES

The anti-IGFBP-3 antibodies (B1 to B10) were evaluated for simultaneous binding (pairing) to IGFBP-3. In brief, each antibody was immobilized onto micro-titre wells at 500 ng/100 µl/well and reacted with IGFBP-3 at 0.0 to 100 µg /L (25 µl/well standards plus 100 µl/well assay buffer). After 1 hour shaking (500-700 rpm) incubation at room temperature (RT), wells were washed four times and reacted with each remaining HRP-labelled anti-IGFBP-3 antibody as above for 30 minutes. Stock HRP-antibodies were diluted about 10,000-fold in the assay buffer and used at 100 µl/well (about 5 ng antibody). After washing, the reaction was developed by 10 minute incubation of the wells with the TMB/H₂O₂ substrate solution (100 µl/well) and addition of the stopping solution as described below. Maximum increase in optical density (OD) of ≤3X background (zero standard signal) between 3X background to 1 OD, between 1 OD to ≤ 2 OD, and ≥2 OD were ranked as indication of no simultaneous binding (pairing) to IGFBP-3, weak pairing, moderate pairing and strong pairing, respectively.

B. COMPETITIVE BINDING OF ANTI-IGFBP ANTIBODIES

The anti-IGFBP-3 antibodies (B1 to B10) were also evaluated for competitive binding to IGFBP-3. In brief, each biotinylated antibody at a predetermined dilution was mixed with increasing concentrations of each remaining unlabelled antibody (0-50 µg/mL) and added (50 µL antibody plus 100 µl assay buffer) to triplicate wells pre-coated with IGFBP-3 (about 75 ng/well). After 2 hours shaking incubation, the wells were washed and developed by 30 minute reaction with HRP-labelled streptavidin (100 µL/well at 2000-fold dilution in the assay buffer) followed by TMB/H₂O₂ and addition of the stopping solution. Decrease in OD of ≤20%, 20-60%, and ≥60% were ranked as indicative of non-interfering, moderately interfering and strongly interfering binding of the antibody pair to IGFBP-3.

C. EFFECTS OF IGF OCCUPANCY ON ANTI-IGFBP BINDING

The effect of IGF occupancy of IGFBP-3 on binding of anti-IGFBP-3 antibodies (B1 to B10) was also evaluated. In brief, ¹²⁵I-IGFBP-3 (1x10⁶ cpm/mL) was mixed with 0.0 to 0.75 µg/mL IGF-I or IGF-II (in ELISA 1 assay buffer), incubated for 2 hours at RT, and 100 µL/well were added in triplicate to anti-IGFBP-3 antibody coated wells. After 2 hours RT incubation, wells were washed three times with dH₂O and counted for bound radioactivity in a Packard RIASTAR gamma counter from PACKARD CANADA (Mississauga, Ontario). An antibody binds at or near the IGF binding site when its IGFBP-3 binding signal (CPM) is decreased by at least 30% in response to IGFBP-3 pre-incubation with IGFs.

D. SIMULTANEOUS BINDING OF ANTI-IGFBP AND ANTI-IGF OR -ALS ANTIBODIES

Native serum IGFBP-3 complex was also evaluated for simultaneous binding to anti-IGFBP-3 antibodies (B1-B10) in combination with anti-IGF-I antibodies (I-1, I-2), anti-IGF-II antibodies (pI-3) or anti-ALS antibodies (pA-1, pA-2) in pair-wise "mix-antibody" sandwich ELISA. In brief, a pool of human serum was prepared by mixing aliquots from 15 different serum samples. Micro-titration strips coated with each antibody were incubated, in quadruplicates, with 50 µL/well of the serum pool or standard matrix buffer and 100 µL/well of the appropriate assay buffer for 2 hours at RT as above. After washing, each quadruplicate set of serum pool/zero standard matrix buffer treated wells were then incubated with 100 µl/well of each of HRP-labelled anti-IGF-I, anti-IGF-II, or anti-ALS antibodies for 30 min at RT. After washing, the reaction was

developed by 10 min incubation with TMB/H₂O₂ substrate and addition of the stopping solution. Increase in OD of $\leq 3 \times$ background (zero standard signal), between $3 \times$ background to 1 OD, and between 1- ≥ 2 OD indicates no binding, moderate binding or strong simultaneous binding of the two antibodies to complexed serum IGFBP-3, respectively. Mix-antibody combinations showing the strongest signal were selected for IGFBP-3 complex ELISA development as described below.

EXAMPLE 4. *EPITOPE MAP OF COMPLEXED IGFBP-3*

Detailed information on epitopes recognized by IGFBP-3 monoclonal antibodies was obtained by evaluating all possible two-site (capture-detection) combinations in pair-wise sandwich ELISA. The spacial distribution of epitopes recognized by each antibody in relation to others was then evaluated in pair-wise competitive ELISA which evaluates binding of a given antibody to IGFBP-3 in the presence of excess amounts of each of the remaining antibodies. The latter provided information on whether an epitope recognized by one antibody was distinct enough to allow non-interfering (independent) binding of a second antibody or whether the epitope recognized were completely or partially overlapping, resulting in binding interferences.

In the third series of experiments, interference in antibody binding to IGFBP-3 by IGF-I or IGF-II was evaluated. This identified antigenic domains at or near the IGF binding site and was assessed by monitoring binding of solid-phase antibodies to IGFBP-3 before and after pre-incubation with radio-labelled IGFs. Finally, the ability of IGFBP-3 antibodies for binding to serum IGFBP-3 complex in pair-wise combination with anti-IGF-I, IGF-II and ALS antibodies was evaluated in "mix antibody" sandwich ELISA. In similar experiments, simultaneous binding of complexed IGFBP-3 to anti-IGFs paired with anti-ALS antibodies was also examined.

As shown in Table 2, assessment of the 10 IGFBP-3 antibodies (B1 to B10) in sandwich ELISA identified 31 of the possible 100 combinations. The binding patterns appear to cluster into four antigenic regions based on reacting antibody combinations and the strength of pairing signal generated. The antibodies were grouped as follows: **Group I** included B5, B6 and B8; **Group II** included B1, B2, B4, and B7; **Group III** included B3; and **Group IV** included B9. Antibodies in the *same* group did not bind *simultaneously* to IGFBP-3 in sandwich ELISA, but demonstrated weak to strong pairing with antibodies in other groups.

Detection-antibody	Capture-antibody									
	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
B1		n	M	n	n	n	n	n	n	n
B2	n		M	n	W	n	n	n	n	n
B3	M	M		M	S	S	W	S	n	n
B4	n	n	M		M	M	n	M	n	n
B5	M	M	S	M		n	n	n	n	n
B6	M	M	S	M	n		M	n	n	n
B7	n	n	M	n	n	M		M	n	n
B8	M	M	S	M	n	n	n		n	n
B9	n	n	S	n	n	n	n	n		n
B10	n	n	n	n	n	n	n	n	n	

Table 2. Pair-wise evaluation of IGFBP-3 antibodies in sandwich ELISA - Simultaneous "two-site" antibody binding patterns to IGFBP-3 are shown. Letters, "n" indicates no simultaneous binding; W, M, S, weak, moderate and strong pairing signal, respectively. B9 and B10, no pairing; B9, strong pairing with B3 only.

Pairing of antibodies in group I and group II appear to depend, to some extent, on whether a given antibody was used for coating or detection. This is presumably due to suboptimal antibody concentrations and/or epitope conformational changes induced by binding of the first antibody to IGFBP-3. The strongest two-site binding signals were generated between antibodies in Group I and Group III (B3). B9 and B10 were unable to form sandwich with each other or with remaining antibodies. B9 demonstrated strong binding to IGFBP-3 only when used as the detection-antibody in combination with B3.

A clearer picture emerged when antibodies were evaluated for binding to solid-phase IGFBP-3 in pair-wise competitive ELISA (Table 3). In these experiments, non-pairing, moderately pairing and strongly pairing antibodies identified in Table 2, appeared to compete with one another strongly, moderately or not-at all, respectively. Only combinations of Group I with Group III antibodies could bind simultaneously to IGFBP-3 without any interference. Again, B9 could also bind strongly to IGFBP-3 when B3 was present.

Competing antibody	Detection-antibody							
	B1	B2	B3	B4	B5	B6	B7	B8
B1		S	M	S	M	M	S	M
B2	S		M	S	M	M	S	M
B3	M	M		M	N	N	M	N
B4	S	S	M		M	M	S	M
B5	M	M	N	M		S	M	S
B6	M	M	N	M	S		M	M
B7	S	S	M	S	M	M		M
B8	M	M	N	M	S	S	M	
B9	n	n	C	n	n	n	n	n
B10	n	n	n	n	n	n	n	n
PSA Ab	n	n	n	n	n	n	n	n

Table 3. Pair-wise evaluation of IGFBP-3 antibodies in competitive ELISA - Simultaneous competitive binding patterns to IGFBP-3 are shown. Letters, "N" indicates non-interfering "simultaneous" binding; S and M, strong and moderate binding interferences, respectively. n, no signal as detection, no interference as competing antibody. C, B9 binding in the presence of B3 only.

Binding of Group I antibodies to IGFBP-3 were significantly inhibited by pre-incubation of IGFBP-3 with IGF-I or IGF-II. This positioned Group I recognition epitopes at or near IGF binding site. In repeat experiments, binding of Group I antibodies to IGFs/IGFBP-3 complexes decreased by more than 50%, whereas activity of Group II, and Group III antibodies as well as the IGFBP-3 polyclonal antibody (pB11) remained relatively unchanged (Table 4, Figure 2). Binding of B9 was about 2-fold higher than that of an irrelevant antibody (anti-PSA) and was not affected by IGF binding to IGFBP-3. B10 was non-reactive as it generated similar signal to that of the anti-PSA antibody.

Mean CPM (SD)			
Antibody	IGFBP-3	IGFBP-3 + IGF-I	IGFBP-3 + IGF-II
B1	9192 (416)	8356 (432)	8736 (264)
B2	9589 (1035)	9633 (1093)	9684 (196)
B3	1617 (151)	16803 (1051)	17913 (82)
B4	5846 (384)	6156 (560)	6235 (78)
B5	5414 (142)	2326 (25)	2060 (214)
B6	5910 (310)	1832 (24)	1435 (64)
B7	4830 (165)	4305 (376)	4698 (49)
B8	6066 (32)	1746 (23)	2110 (39)
B9	1257 (141)	960 (71)	1269 (69)
B10	706 (25)	495 (50)	602 (63)
pB111	28466 (902)	30485 (503)	30767 (357)
PSA Ab	594 (115)	457 (38)	483 (13)

Table 4. Inhibition of Antibody binding to IGFBP-3 by IGF-I and IGF-II - Binding of iodinated rIGFBP-3 to antibody coated wells examined with or without pre-incubation with IGF-I or IGF-II. Values are the mean (SD) of three measurements.

In two-site "mix-antibody" ELISA (anti-IGFBP paired with anti-ALS or anti-IGF), antibodies in Group II and Group III demonstrated simultaneous binding to the naturally occurring (native) serum IGFBP-3 complex in combination with anti-IGF-I (I-1) or an anti-ALS (pA-1) antibodies used for detection. The strongest pairing signal was observed with the B2/pA-1 antibody pair and the B3/I-1 antibody pair. No simultaneous binding to serum IGFBP-3 complex was observed when the anti-IGF-I antibodies were evaluated against anti-ALS antibodies in all possible mix-antibody combinations. Bindings of other possible mix-antibody combinations were similar to that involving the negative control anti-PSA antibody (Table 5).

Capture-antibody	Detection-antibody				
	I-1	I-2	pI-3	pA-1	pA-2
B1	W	n	n	W	n
B2	M	n	n	M	n
B3	W	n	n	M	n
B4	W	n	n	W	n
B5	n	n	n	n	n
B6	n	n	n	n	n
B7	W	n	n	W	N
B8	n	n	n	n	n
B9	n	n	n	n	n
B10	n	n	n	n	n
PSA Ab	n	n	n	n	n

Table 5. Pair-wise evaluation of IGFBP-3 antibodies in combination with anti-IGF-I or anti-ALS antibodies for binding to IGFBP-3 complexes - Simultaneous "two-site" mix-antibody binding patterns to serum IGFBP-3 complex are shown. Letters, "n", no simultaneous binding; W, M, S, weak, moderate and strong pairing signals, respectively.

The binding characteristics of the four groups are summarized as follows:

Group I: IGFBP antibodies (B5, B6 and B8) This group of antibodies recognized epitopes that mapped at or near the IGFBP-3 ligand binding site as defined by their binding inhibition in response to IGFBP-3 pre-incubation with the IGFs. These antibodies could not be distinguished from each other as they did not bind simultaneously to IGFBP-3 in sandwich ELISA and strongly competed with each other in competitive binding assays.

By western immunoblot of various IGFBP-3 fragments, two of Group I antibodies (B5 and B8) have been recently shown to recognize epitopes at both N-terminal (IGFBP-3₁₋₉₇) as well as C-terminal (IGFBP-3₂₀₀₋₂₆₄) regions of IGFBP-3, whereas B6 was found to react only with the N-terminal (IGFBP-3₁₋₉₇) fragment (44). The result of the present study seems to indicate binding of B6 to the C-terminal as well as N-terminal regions of IGFBP-3. B6 strongly inhibited binding of B5 and B8 to IGFBP-3 in pair-wise competitive ELISA, but as with B5 and B8, demonstrated strong "non-overlapping" sandwich formation with B3.

It is also possible that B6 binds to N-terminal sequences that forms part of the IGFBP-3 ligand (IGF) binding site. As the IGF binding site of IGFBP-3 is thought to involve both N- as well as C-terminal sequences, antibodies that bind to such conformational epitopes could compete strongly for binding to the native molecule, but differently to denatured fragments in western immunoblot analysis. However, our findings of ligand binding site specificity for antibodies that reportedly bind to both N- and C-terminal of IGFBP-3 (44) is consistent with the notion that both N- and C-terminal sequences of IGFBP-3 contribute to the formation of IGF-binding site (44, 45).

Group II: IGFBP antibodies (B1, B2, B4, B7) - Although epitopes recognized by Group II antibodies overlapped with those specified by Group-I antibodies, the immuno-reactivity of Group II antibodies was not affected by IGF binding to IGFBP-3. The antigenic cluster recognized by Group II antibodies was therefore, physically mapped to an area on the molecule distant from the IGFBP-3 ligand binding site. Again Group II antibodies could not be distinguished from each other as they appeared to bind to overlapping epitopes. Consistent with our findings is the reported specificity of Group II antibodies for the intermediate sequences of IGFBP-3 (IGFBP-3₉₈₋₁₅₉) as evaluated by western immunoblot analysis (44).

Group III: IGFBP Antibody (B3) Only one antibody defined a third epitope. The determinant for this antibody also overlapped with those of Group II, but was distinct from Group I antibodies. B3 formed strong non-competing sandwich assays with antibodies in Group-I. Interestingly, this antibody have been found to recognize an epitope within the N-terminal region of IGFBP-3 (IGFBP-3₁₋₉₇) (44), which according to our findings must not overlap with those of N-terminal IGF-binding region.

Group IV: IGFBP Antibody (B9) A fourth antigenic epitope recognized by B9 appeared to be a distinct conformational epitope that was accessible only after binding of B3 to IGFBP-3. In capture-detection-antibody binding ELISA, Group II and Group III antibodies could bind simultaneously to the native serum IGFBP-3 complexes in combination with the I-1 or pA-1 antibody used for detection. The strongest pairing signal was obtained for the B3/I-1 (ELISA-1) and B2/pA-1 (ELISA-2) combinations. This indicates that even in the native IGFBP-3 ternary complexes, antigenic domains on IGF-I as well as ALS are accessible for antibody binding and can participate in two-site "capture-detection" antibody formation. Consistent with these findings, are the recent observations that binding of rIGF-I to solid-phase IGFBP-3 was detectable by C-terminal specific (D-domain, residues 63-70) anti-IGF-I mouse monoclonal antibodies in

sequential binding experiments (46).

The lack of simultaneous "two-site" binding of the above I-1 and pA-1 to serum IGFBP-3 complex suggests close binding proximity of the N-terminal part of ALS and IGF binding site of IGFBP-3 (Figure 1). This observation may provide an explanation for the reported finding that ALS binding to IGFBP-3 is an important modulator of IGFBP-3 affinity for the IGF peptides (47). Similar to the effect of antibody B3 on the immuno-reactivity of antibody B9 described above, proximal ALS binding to IGFBP-3 ligand binding site could cause conformational changes, leading to enhanced IGF binding affinity of IGFBP-3.

EXAMPLE 5. *Methods: ELISA-1 and -2.*

A. ELISA-1

Among possible anti-IGFBP-3/anti-IGF-I combinations, the anti-IGFBP-3 B3 and anti-IGF-I I-1 generated strongest binding signal to serum IGF-binding protein complex when used as capture and detection-antibodies, respectively. The ELISA protocol was optimized as described previously (37, 41).

In the assay, standards or serum samples (0.025 mL of 10-40 fold diluted in the assay zero standard matrix buffer) were added in duplicate to antibody coated wells, followed by addition of the ELISA 1 assay buffer (0.10 mL) and 1 hour incubation at room temperature with continuous shaking. The wells were washed five times and incubated with 0.1 mL/well of the anti-IGF-I-HRP conjugate (diluted in the assay buffer to approximately 0.1-0.25 mg/L) for 1 hour at room temperature. The wells were washed five times with the wash solution, 0.1 mL of the TMB/H₂O₂ substrate solution added for an additional 10 min incubation at room temperature. Stopping solution (0.1 mL) was then added and absorbance measured by dual wavelength measurement at 450 nm with background wavelength correction set at 620 nm.

ELISA data were analysed using data reduction packages included in the LABSYSTEMS microplate reader with cubic spline (smoothed) curve fit. Other statistical analyses were performed using the MICROSOFT EXCEL 97 STATISTICAL PACKAGE by MICROSOFT CORPORATION (USA) on an IBM clone Pentium computer. Descriptive data are presented as the mean and SD unless otherwise specified. Linear-regression analysis was performed by the least-squares method and correlation coefficients were determined by the Pearson method.

B. ELISA-2

Among possible anti-IGFBP-3/anti-ALS combinations, the anti-IGFBP-3 B2 and anti-ALS pA-1 generated the strongest binding signal to serum IGF-binding protein complex when used as capture and detection-antibodies, respectively. The assay protocol was exactly as described above, except that the optimal sample and assay buffer volumes were 25 μ l and 50 μ l/well, respectively.

C. VALIDATION OF ELISA-1 AND -2.

For validation of IGFBP-3 complex ELISA-1 and ELISA-2, appropriately diluted fresh serum samples were used. In both assays, the lower limit of detection (sensitivity) was determined by interpolating the mean plus 2SD of 12 replicate measurements of the negative control (zero standard matrix buffer). The intra-assay correlation coefficients (CVs) were determined by replicate analysis (n=12) of 3 samples at IGFBP-3/IGF-I levels of 8.6-24.3 AU/L, and IGFBP-3/ALS levels of 6.7-18.7 AU/L; interassay CVs by duplicate measurement of appropriate samples in 7-9 separate runs. Recovery was assessed by adding 50 μ L of samples containing different IGFBP-3 complex levels to 450 μ l of 10-fold diluted serum samples followed by analysis. Percent recovery was determined by comparison of the amount of added IGFBP-3 complex to the amount measured after subtracting the endogenous IGFBP-3 complex level. Linearity was tested by analysing serum samples diluted first 10-fold, then serially 2-16 fold in the zero standard matrix buffer. IGFBP-3 complex ELISA specificity was analysed by assaying IGF-I (up to 300 μ g/L), IGF-II (up to 3000 μ g/L), IGFBP-1, 2, 4-6 up to 500 μ g/L, and IGFBP-3 up to 4.3 mg/L.

To evaluate IGFBP-3 complex stability, and thus practical use of the assay, aliquots of fresh serum samples (n=3) were stored at RT, 4°C, and -20°C and then analysed on day 0, 2 and 3 of storage by ELISA-1 and ELISA-2 after 10-fold dilution in the assay zero standard matrix buffer. On the day of analysis, samples were assayed against a new set of standards freshly prepared from a frozen (at -20°C) aliquot of the stock standard serum pool. Aliquots of a set of standards were also stored at the above temperatures and similarly analysed.

ELISA-1 and -2 results were compared with results obtained from prior art assays. IGF-I, IGF-II, IGFBP-3 and ALS were analysed by immunoassay kits manufactured by DSL (Webster, TX). These assays are based on non-competitive ELISA principles performed in antibody coated micro-wells (15, 37, 38, 39) and horseradish peroxidase (HRP) labelled detection-antibodies as

described (41).

IGF-I and IGF-II were assayed by the ACTIVE™ Non-Extraction IGF-I and IGF-II ELISA (DSL, Webster) which incorporates a sample pre-treatment step to dissociate IGF-I from IGFBPs prior to analysis (43). The sample pre-treatment step involves mixing 20 µl of sample with 1.0 mL of IGF-I acidification buffer followed by 30 min room temperature incubation, and addition of 1.0 mL of the neutralization buffer. The final sample preparation dilution factor was 101-fold and 20 µl of the treated sample is used for IGF analysis. The IGF ELISA kits each have a total incubation time of less than 3 hours and an overall imprecision of less than 10%.

The ACTIVE™ IGFBP-3 ELISA (DSL, Webster) incorporates a 101-fold sample pre-dilution and uses 25 µl of the pre-diluted sample for IGFBP-3 analysis. The assay has a total incubation time of about 3 hours, a standard range of 2-100 µg/L (0.20-10 mg/L after correction for sample dilution factor) and an overall imprecision of less than 10% (39).

The ACTIVE™ Total ALS ELISA (DSL, Webster) incorporates a 101-fold sample pre-treatment step that would result in unfolding of both complexed and uncomplexed ALS, thus allowing measurement of the total ALS levels (15). The sample pre-treatment step involves mixing of 10 µl of sample with 1.0 mL of the sample pre-treatment buffer followed by 30 minute room temperature incubation. The final sample preparation dilution factor was 101-fold and 20 µl of the treated sample is used for Total ALS analysis. The assay has a total incubation time of about 2 hours, a standard range of 6-600 µg/L (0.6-60 mg/L after correction for sample pre-treatment dilution factor) and an overall imprecision of less than 10%.

Absorbance of ELISAs were measured with the LABSYSTEMS MULTISKAN MULTIOFT microplate reader by LABSYSTEMS, (Helsinki, Finland).

EXAMPLE 6. Results: ELISA-1 and ELISA-2

A. ELISA-1 AND -2.

Based on the above epitope mapping, novel immunoassays for quantisation of circulating IGFBP-3 complexes were developed. Both the IGFBP-3/IGF-I ELISA-1 and the IGFBP-3/ALS ELISA-2 involve a two-site noncompetitive (sequential) immunoreaction and are based on solid-phase anti-IGFBP-3 B3 or B2 antibody paired with detection anti-IGF-I I-1 or anti-ALS pA-1 antibodies, respectively.

Optimized protocols were established by evaluating the effects of various technical

manipulations on the analytical performance of the assays as previously described (37, 41). A coating antibody concentration of 10 mg/L, a detection-antibody concentration of about 0.1-0.25 mg/L, a 60 minute first and second step room temperature incubations, and a 10 minute substrate development step were selected. Among the variables examined, composition of the assay buffer and coating antibody concentrations had the most obvious effect on sensitivity and dynamic range of the assays.

A typical standard curve and performance characteristics of ELISA-1 and ELISA-2 are summarized in Figure 3 and Table 6. Addition of IGF-I (up to 300 µg/L), IGF-II (up to 3000 µg/L), IGFBP-2 and IGFBP-4-6 (up to 500 µg/L), and IGFBP-3 (up to 4.2 mg/L) to the zero standard matrix buffer did not show any cross-reactivity.

Assay Parameter	ELISA-1	ELISA-2
Detection limit, AU/mL	0.12	1.13
Standard range, AU/mL	0.78 - 50	3.13 - 50
Intrassay CV, %	3.5 - 6.6	4.3 - 7.6
Interassay CV, %	4.8 - 9.7	5.3 - 10.6
Recovery of added complex, %	84 + 4.6	90 + 9.2
Recovery after dilution, %	107 + 11.3	96 + 12.4

Table 6. Complex IGFBP-3 ELISA-1 and -2 validation data - No cross reactivity was detected with IGF-I, IGF-II, IGFBP-1 to -6, and ALS.

Serum IGFBP-3 complexes were analysed in replicate aliquot of samples stored at RT, 4°C and -20°C. As measured by both ELISA-1 and ELISA-2, IGFBP-3 complexes demonstrated high stability at all temperatures for up to 3 days of storage, and recoveries at 4°C and -20°C were at least 85% of the day 0 values (Figure 4, 5). Serum-based standards stored and analysed as above demonstrated similar stability, and IGFBP-3 complexes in a pool of fresh sera stored at -20°C demonstrated at least 2 months stability.

Despite the complexity of their design, the ELISA-1 and ELISA-2 assays for IGFBP complex demonstrated acceptable analytical performance characteristics. The finding of relatively high stability of the IGFBP-3 complexes at various temperature and even in diluted form was unexpected and was instrumental in the success of the development of the assays. The demonstrated linearity of the assays in response to sample dilution may suggest measurement of

only the tightly bound IGFBP-3 ternary complexes. Antibody binding and sample dilution may induce IGF dissociation and, thus removal of loosely bound complexes.

B. COMPARISON WITH PRIOR ART ASSAYS

Plasma samples from subjects with untreated GHRD (n = 11) and age-matched normal subjects (n = 16), and serum samples from adults with acromegaly (n = 8) or GHD (n = 5) were simultaneously analysed by ELISA-1 and ELISA-2 and for IGF-I, IGF-II, IGFBP-3 and total ALS levels.

Regression analysis of data showed high degree of correlations between IGFBP-3 complex ELISA-1 and -2 versus IGFs, IGFBP-3 and total ALS levels (Table 7, and Figures 6-9). The best overall correlations were observed in comparisons with IGF-I levels, while correlation against IGF-II levels were relatively poor. The flattened "low-end" appearance of correlation graphs are due to significant differences in the relative levels of the various analytes as a function of age (Figure 10) as well as clinical conditions, and not necessarily due to poor low end correlations.

In fact, correlations in the GHRD range was the same as those involving all sample values.

X	Y	Slope	intercept	R2	p
IGF-I	ELISA-1	1.03	25.2	0.92	<0.005
	ELISA-2	0.39	11.3	0.96	<0.005
IGF-II	ELISA-1	245	1.63	0.32	0.124
	ELISA-2	104	0.66	0.37	0.066
IGFBP-3	ELISA-1	173	194	0.84	<0.005
	ELISA-2	64.9	68.1	0.84	<0.005
ALS	ELISA-1	19.35	152	0.87	<0.005
	ELISA-2	49.6	7.1	0.84	<0.005

Table 7. Correlation matrix for IGFBP-3 complex ELISA comparison with ALS, IGF-I, IGF-II and IGFBP-3 - X, comparative ELISA methods. Y, ELISA-1, IGFBP-3/IGF-I; ELISA-2, IGFBP-3/ALS.

Although the number of normal samples in various age groups were small, the overall pattern of IGFs, IGFBP-3 and ALS levels versus age were similar to those previously reported, with levels showing significant rise during pubertal age (21, 34, 35). As expected, IGFBP-3

complex levels were similarly affected by age. However, IGF-I based complexes measured by ELISA-1 showed the widest variations, being lowest in the youngest and oldest age groups and highest during pubertal age (Figure 10).

In plots of mean analyte levels as a percentage of the corresponding mean of normal values, the mean of IGFBP-3 complex levels measured in GHRD and GHD subjects by ELISA-1 were less than 8% and 2% of the mean of normal values, respectively. The next largest differences were observed for IGF-I levels; the mean IGF-I levels in GHRD and GHD subjects were less than 13% and less than 24% of the mean of normal values (Figure 11). In comparative distribution plots of the individual values, IGFBP-3 complex levels by ELISA-1 demonstrated similar if not better discrimination between the various sample groups, particularly between GHRD, normals and GHD subjects (Figure 12).

In several experiments involving randomly selected samples, the IGFBP complex ELISAs showed significant correlation with IGF-I, IGFBP-3 and ALS levels. This was subsequently confirmed by the preliminary clinical evaluations involving samples from subjects with GHRD, their age-matched normal controls, and specimens from acromegalic and GHD adults. Overall, both ELISA-1 and ELISA-2 demonstrated high correlation with IGF-I, IGFBP-3 and ALS levels. This supports the reported observations that most of circulating IGFs and IGFBP-3 are primarily present in the ternary protein complex and that IGF availability may be a key determinant of ternary complex formation (4-12).

Of interest was the observation that the IGFBP complexes measured by ELISA-1 showed the widest relative variations as a function of age. The IGFBP complex levels of the individual samples were lowest in the younger age group and highest in the older, pubertal range subjects (Figure 10). Similarly, in comparative distribution plots of the individual sample values, ELISA-1 demonstrated a similar, if not better discrimination between the various sample groups (Figure 12). The relatively lower IGFBP complex measured in younger age group by ELISA-1 may suggest availability of proportionally higher amount of IGF-I in the free (or dissociable) form in this age group, as has been recently reported for early infancy (48).

C. CONCLUSIONS

The data presented here establish that the antibodies described herein can be selected on the basis of their epitope maps and employed in a successful capture-detection ELISA assay

format. Additional antibody mapping can be performed in the manner described here. In particular, it would be beneficial to identify anti-IGFBP antibodies whose binding is unaffected by IGF-I or ALS binding. Such antibodies would allow the assay of total IGFBP-3 without regard for the degree of complex formation.

5 In addition to the two antibody pairs exemplified above (ELISA-1 and -2), other antibody pairs or even triplets can be optimized in a manner similar to that described herein. Hence, the above examples are not to be construed as limiting, but rather as illustrative of the many antibody combinations that can be employed in a capture-detection format.

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CLAIMS**What is claimed is:**

1. A kit for the assay of an IGFBP-complex,
said kit comprising a capture-antibody and a detection-antibody,
5 wherein said capture-antibody and said detection-antibody do not bind to
overlapping epitopes and do not interfere with the formation of said IGFBP
complex.
2. The kit of claim 1,
10 wherein said capture-antibody is bound to a solid support and said detection-antibody is
coupled with a label.
3. The kit of claim 2, wherein the capture-antibody and a detection-antibody are
selected from the group consisting of B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, pB11, I-1, I-2,
15 pI-3, pA-1 and pA2.
4. The kit of claim 1 or 2, wherein the capture-antibody is B3 and the detection-
antibody is I-1.
- 20 5. The kit of claim 1 or 2, wherein the capture-antibody is B2 and the detection-
antibody is pA-1.
6. The kit of claim 2, 3, 4, or 5, further comprising a label detection means, a binding
buffer, a wash buffer, a detection buffer and a control standard.
- 25 7. A method of detecting IGFBP complex in a body fluid, said method comprising
a) contacting a solid phase coupled to a capture-antibody with a body fluid to
capture an IGFBP complex;
b) washing the solid phase with a first wash buffer;
30 c) contacting the solid phase with a detection-antibody coupled to a label;
d) washing the solid phase with a second wash buffer; and

e) detecting said label remaining with said solid phase after said washing step.

8. The method of claim 7, further comprising acid treating said IGFBP complex.

5 9. The method of claim 7, wherein the capture-antibody and a detection-antibody are selected from the group consisting of B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, pB11, I-1, I-2, pI-3, pA-1 and pA2.

10 10. The method of claim 7, wherein the capture-antibody is an IGFBP-3 monoclonal antibody and the detection-antibody is an IGF or ALS monoclonal antibody.

11. The method of claim 7, wherein the label is horseradish peroxidase or biotin.

15 12. The method of claim 7, wherein the capture-antibody is B3 and the detection-antibody is I-1 or the capture-antibody is B2 and the detection-antibody is pA-1.

13. The method of claim 12, wherein the label is horseradish peroxidase or biotin and the solid support is a micro-titre well plate.

20 14. A method of monitoring the effect of treatment designed to influence growth hormone status said method comprising the collection of a body fluid and testing said body fluid with the method of claim 7.

25 15. A method of determining the growth hormone status of an individual, said method comprising the collection of a body fluid and testing said body fluid with the method of claim 7.

16. The method of claim 15, wherein the individual is human, bovine, porcine, equine, canine, feline, or ovine.

30 17. A method of determining the growth hormone status of an individual with a growth-related disease or a susceptibility to a growth-related disease, comprising:

a) collecting a body fluid of said individual;

- b) contacting a solid phase coupled to a capture-antibody with said body fluid to capture an IGFBP complex;
- c) contacting said solid phase with a detection-antibody coupled to a label; and
- d) detecting said label, wherein the amount of said label correlates with the growth hormone status of said individual.

5

18. The method of claim 17, wherein said growth-related disease is selected from the group consisting of hypoglycaemia, diabetes, deficient nutritional states, breast cancer, prostate cancer, immune deficiencies, fetal growth retardation, gigantism, acromegaly, hyperpituitarism, pituitary dwarfism, GH deficiency, GH excess, GH receptor defect, thyromegalia and Alzheimer.

10

19. The method of claim 17, wherein the individual is human, bovine, porcine, equine, canine, feline, or ovine.

20. A method of monitoring the treatment of an individual with a growth-related disease or susceptibility to a growth-related disease, comprising:

15

- a) contacting a solid phase coupled to a capture-antibody with a body fluid of an individual with a growth-related disease to capture an IGFBP complex;
- b) contacting said captured IGFBP complex with a detection-antibody coupled to a label;
- and
- e) detecting said label.

20

21. The method of claim 20, wherein said growth-related disease is selected from the group consisting of hypoglycaemia, diabetes, deficient nutritional states, breast cancer, prostate cancer, immune deficiencies, fetal growth retardation, gigantism, acromegaly, hyperpituitarism, pituitary dwarfism, GH deficiency, GH excess, GH receptor defect, thyromegalia and Alzheimer.

25

Figure 1

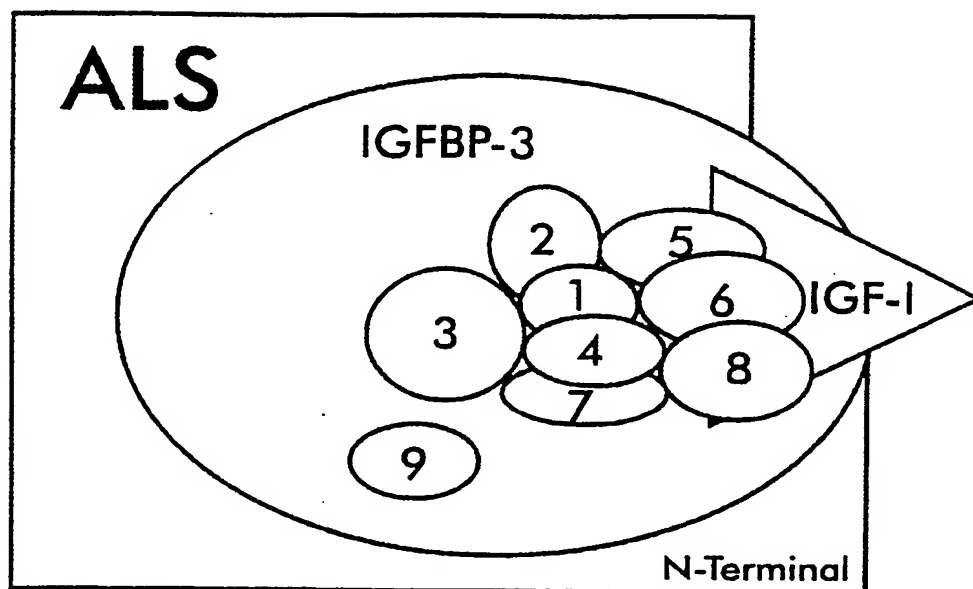


Figure 2

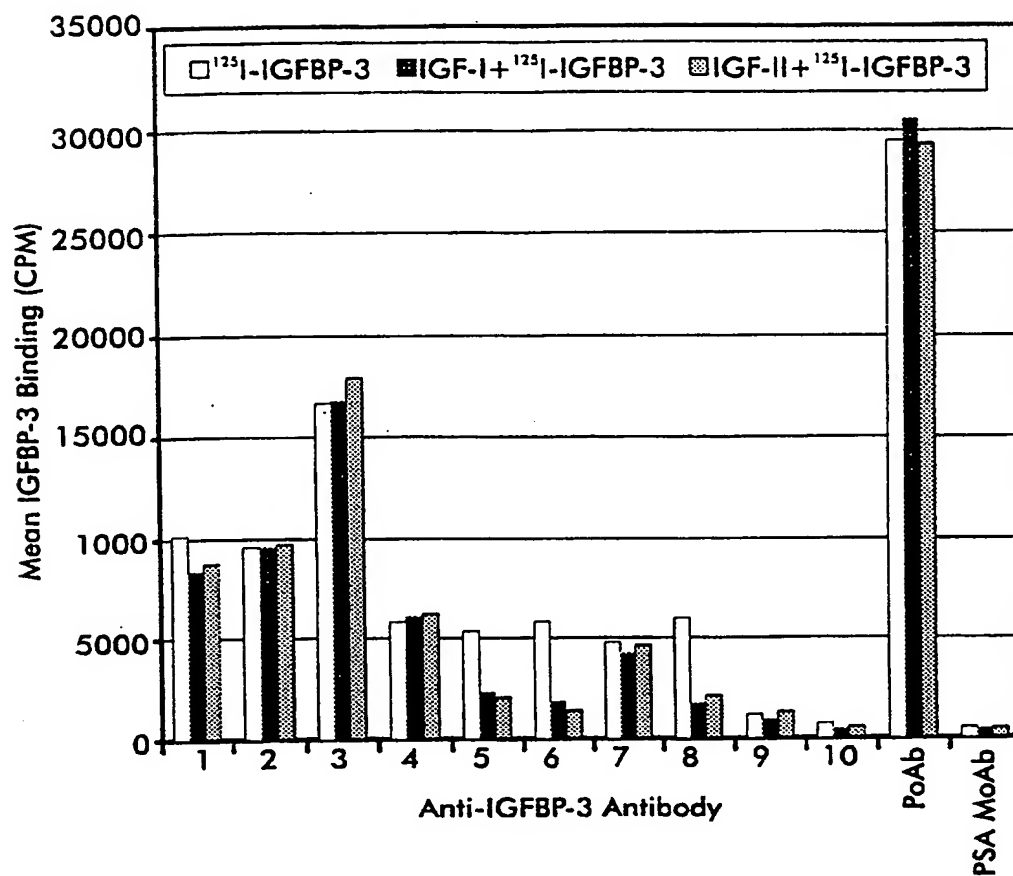


Figure 3a

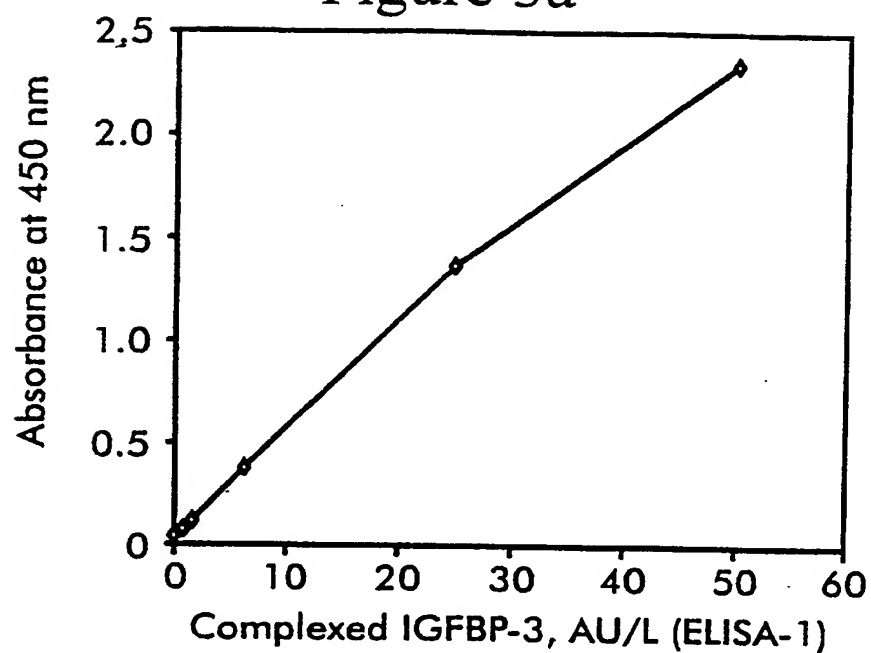


Figure 3b

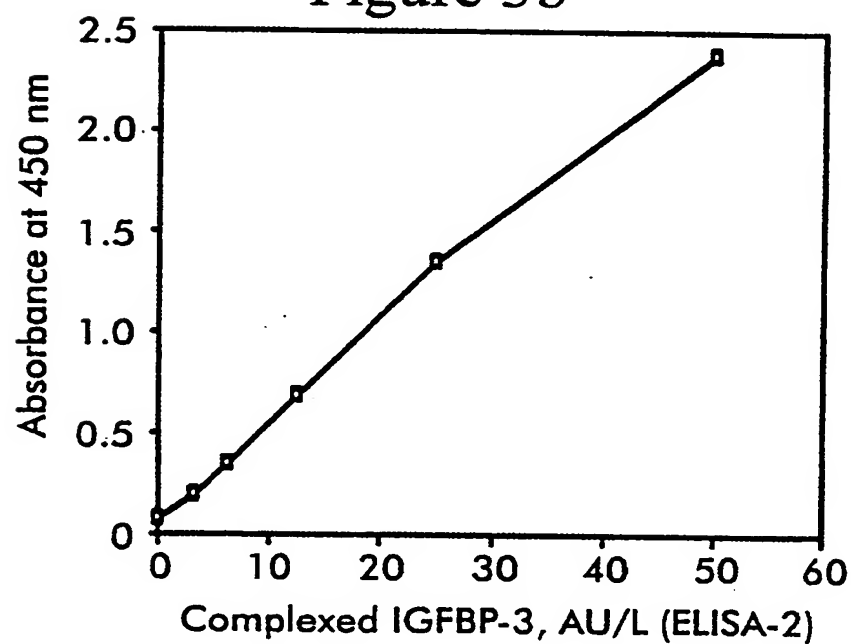


Figure 4a

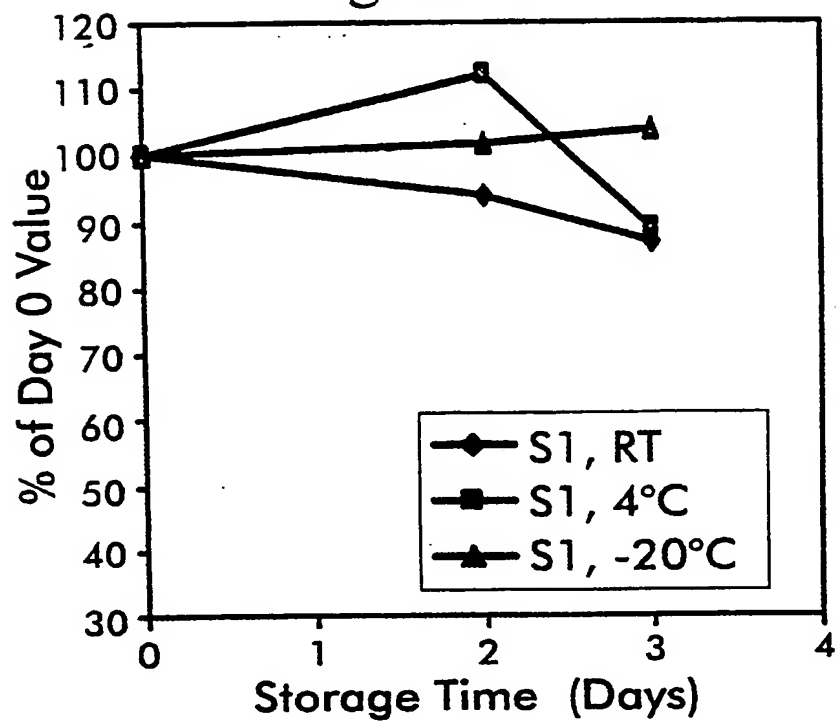


Figure 4b

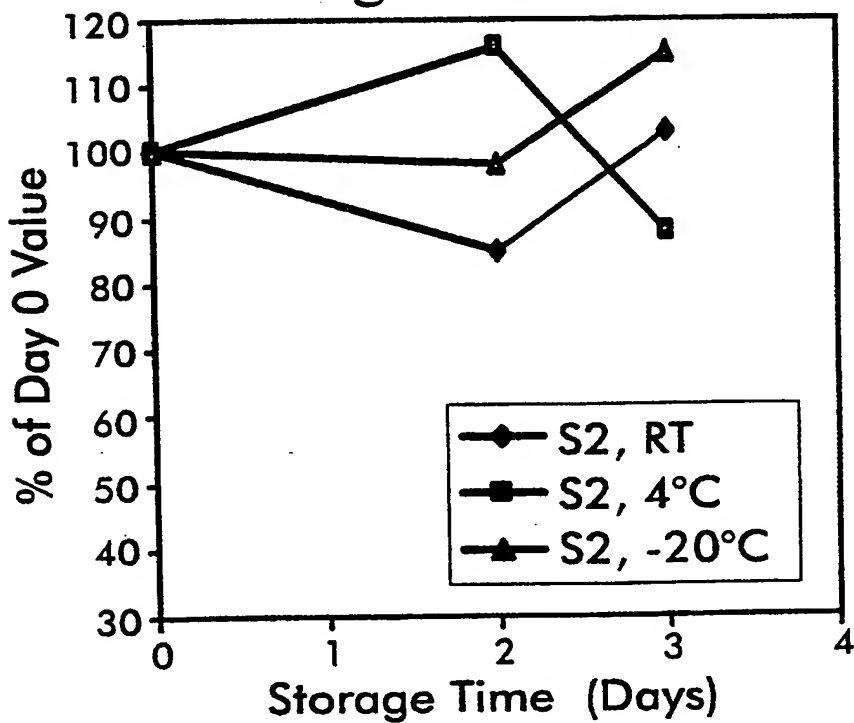


Figure 4c

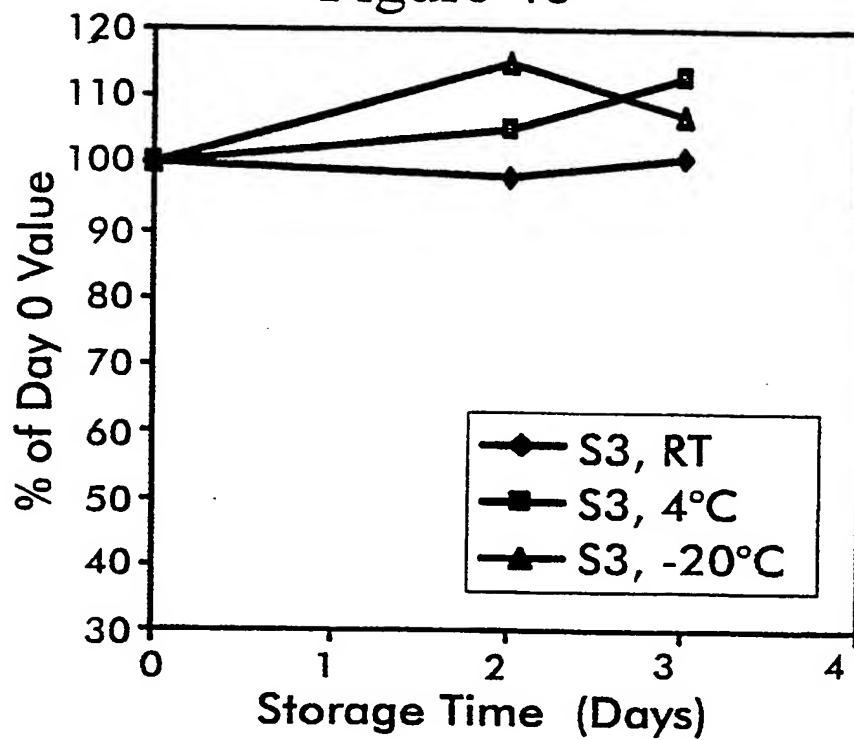


Figure 5a

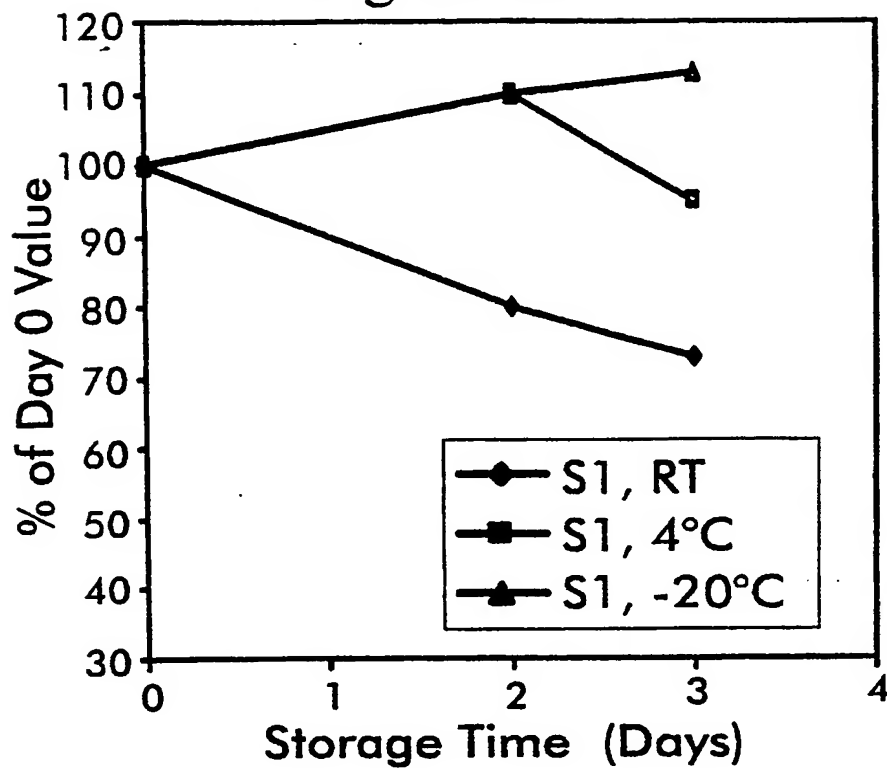


Figure 5b

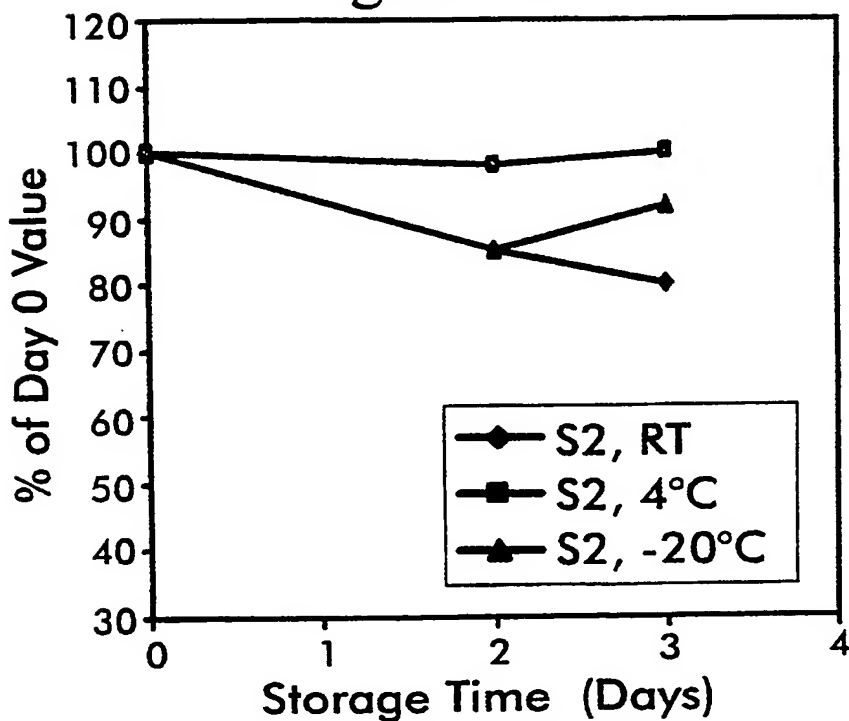


Figure 5c

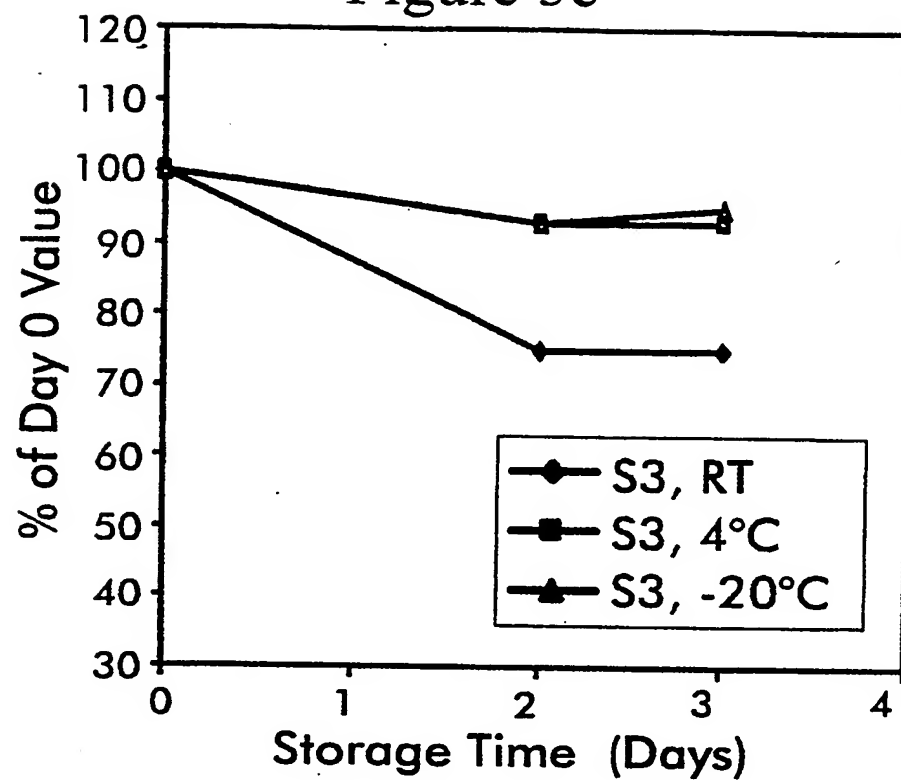


Figure 6a

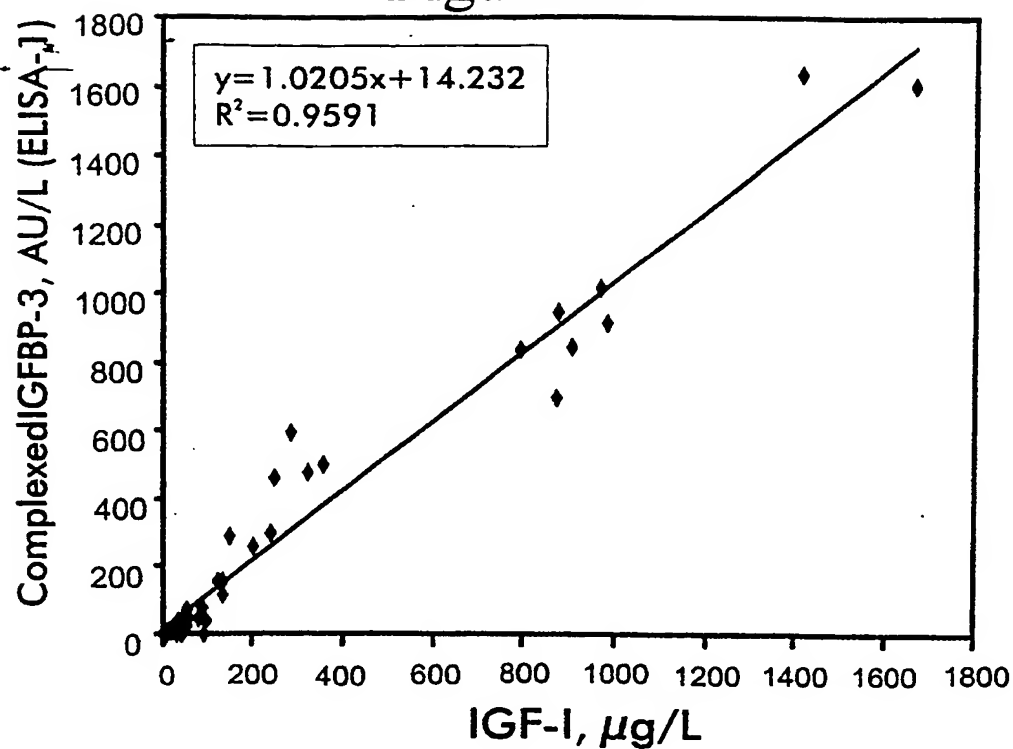


Figure 6b

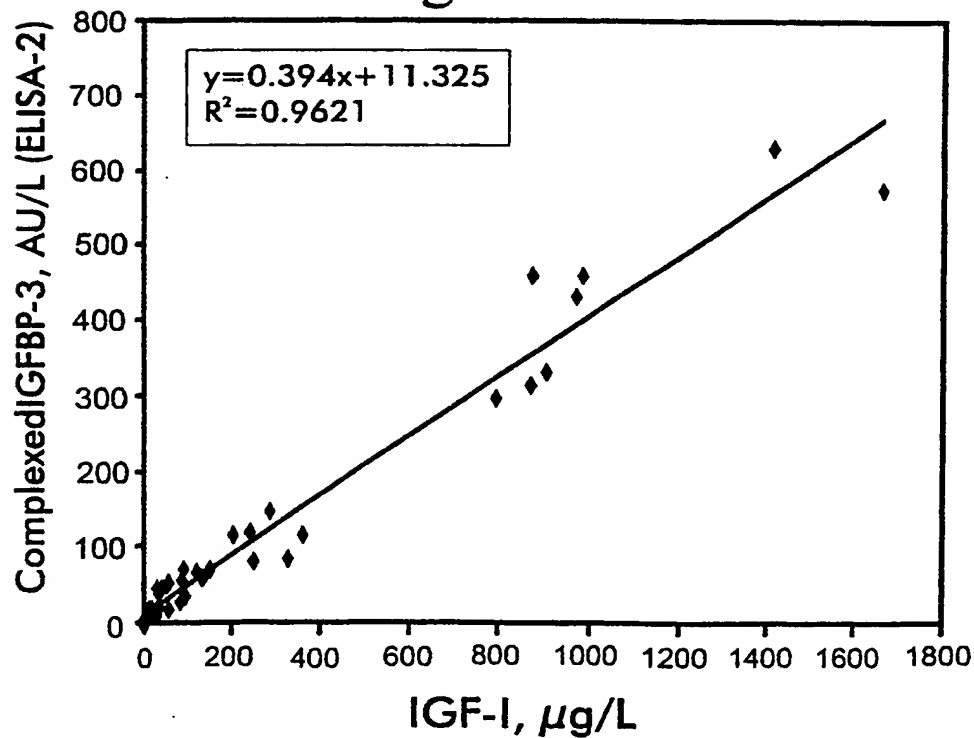


Figure 7a

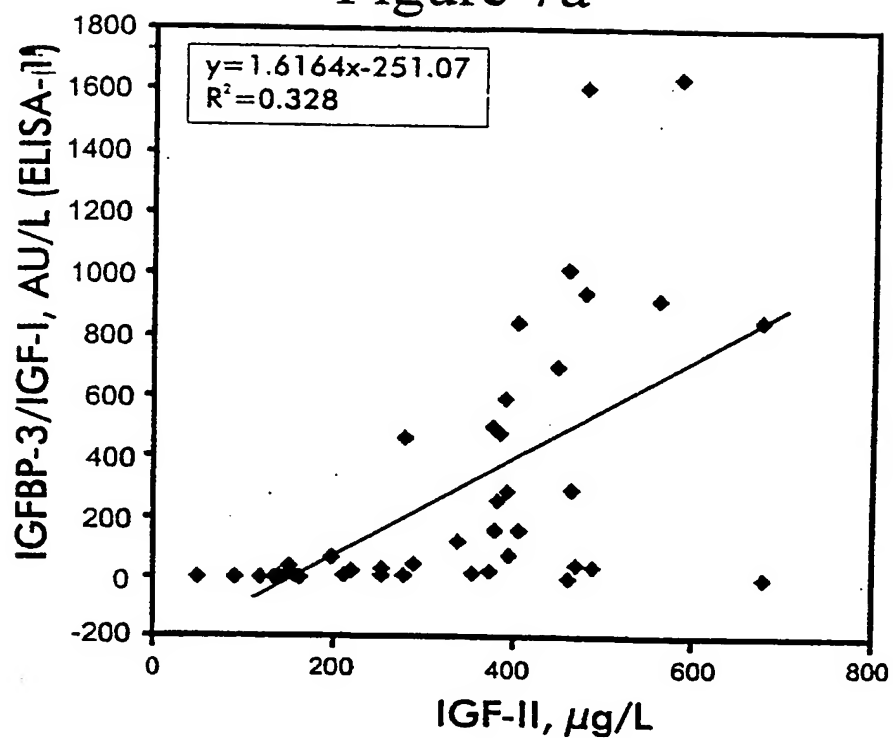


Figure 7b

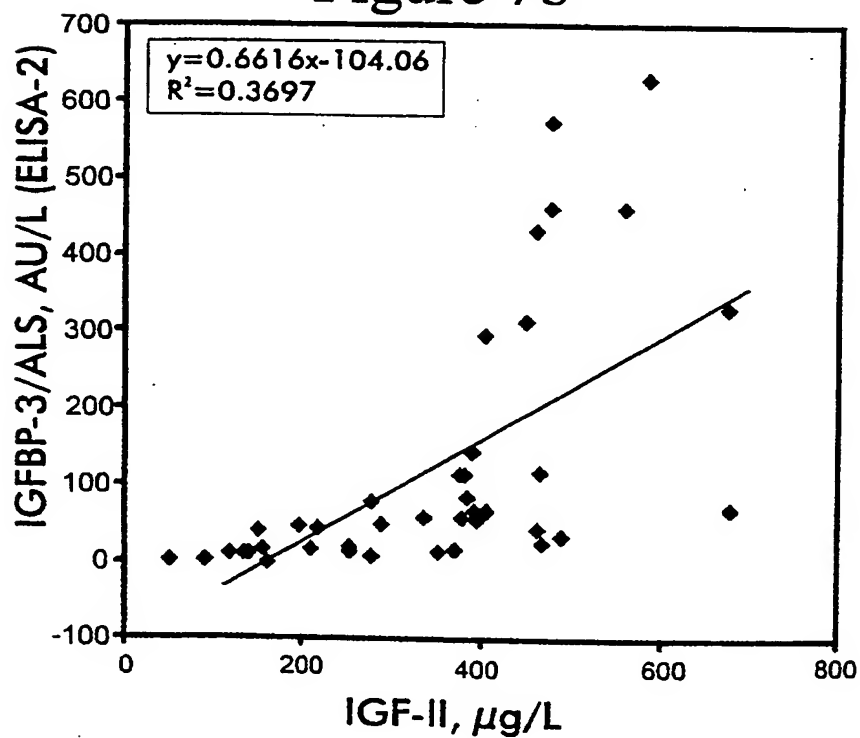


Figure 8a

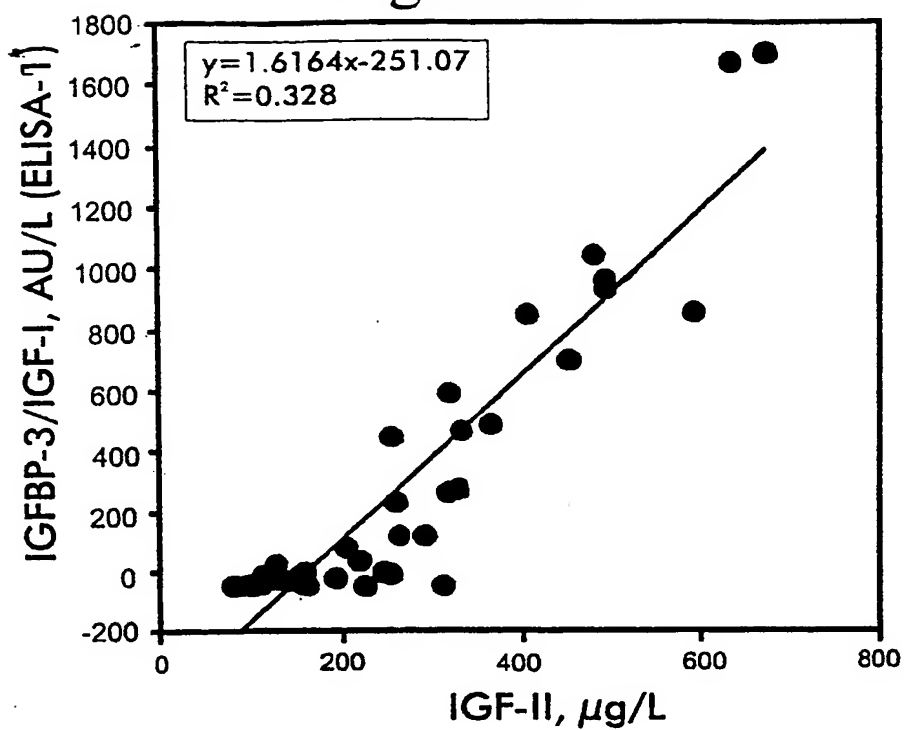


Figure 8b

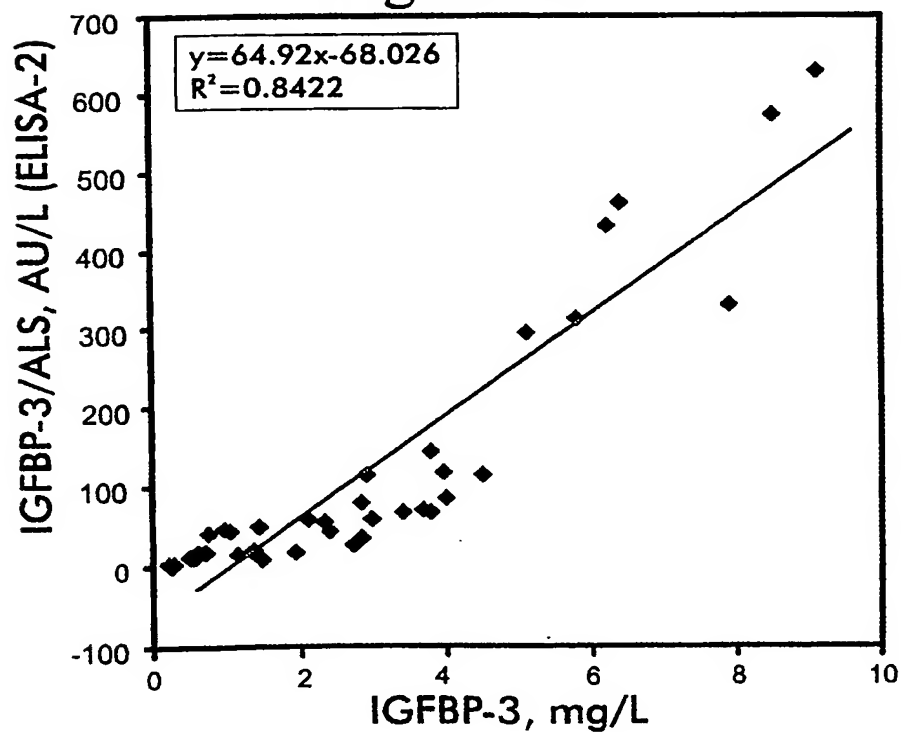


Figure 9a

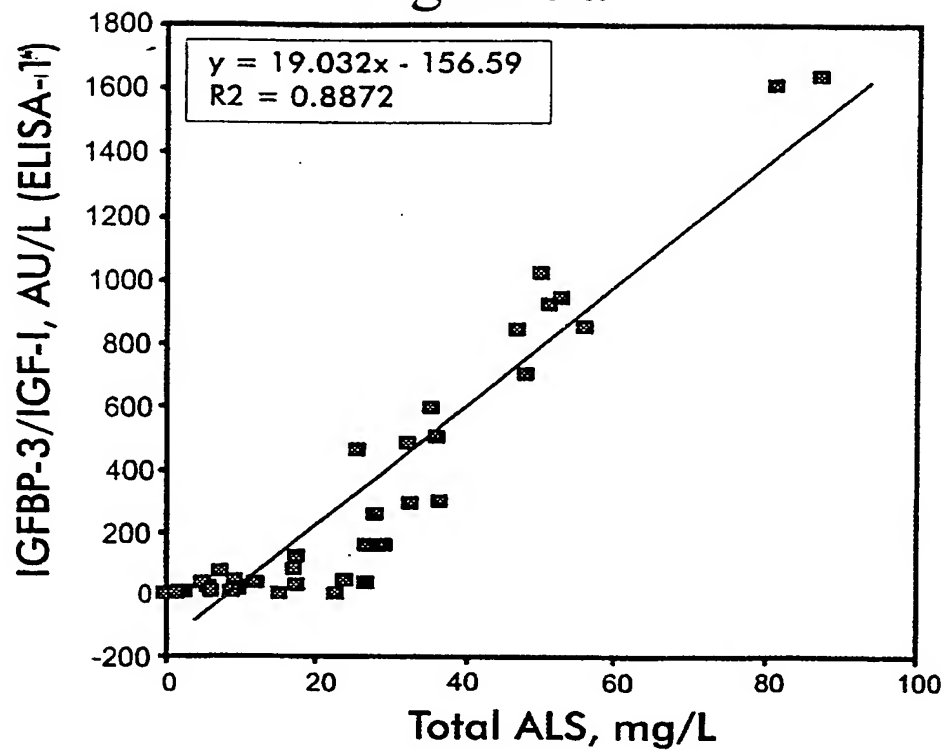


Figure 9b

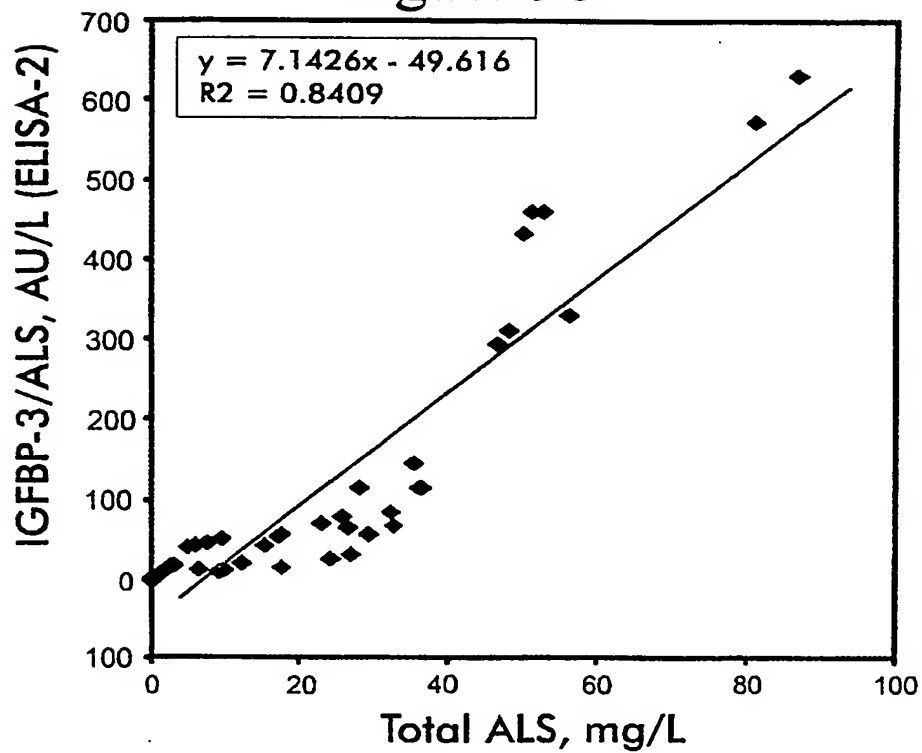


Figure 10

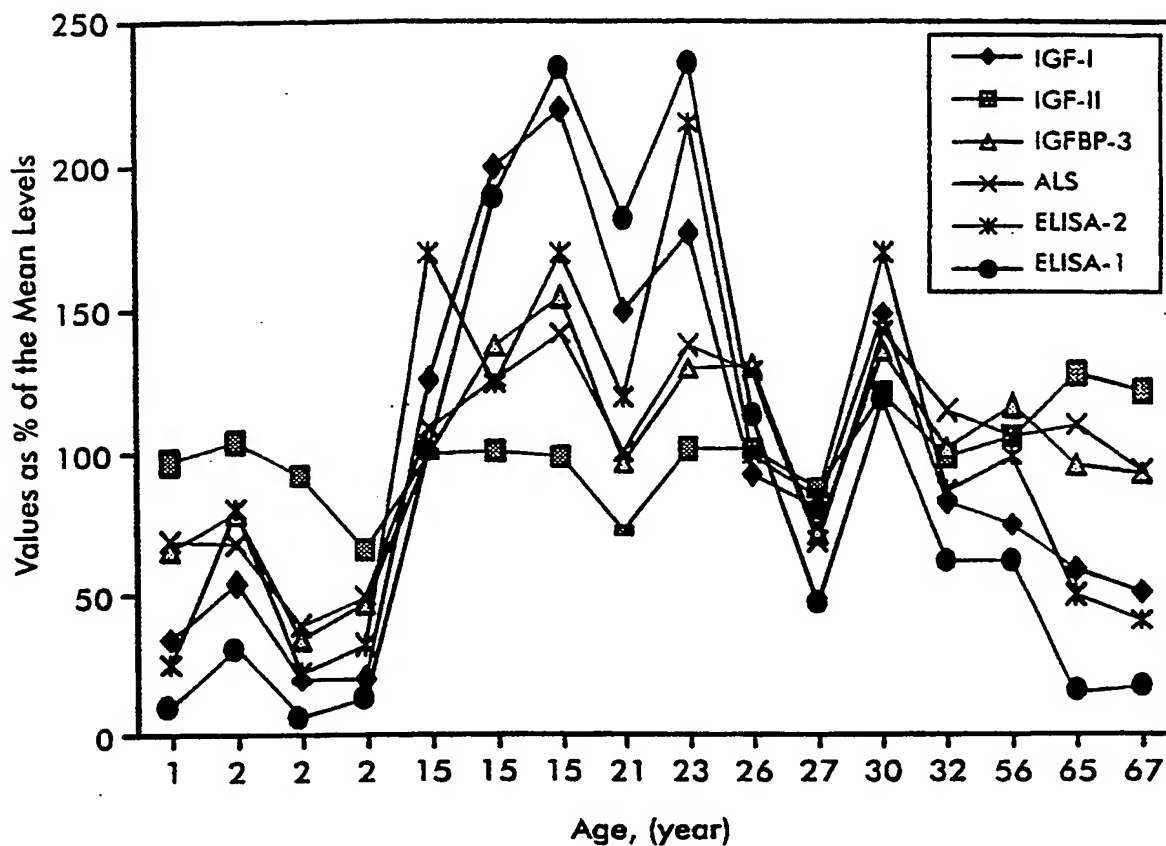


Figure 11

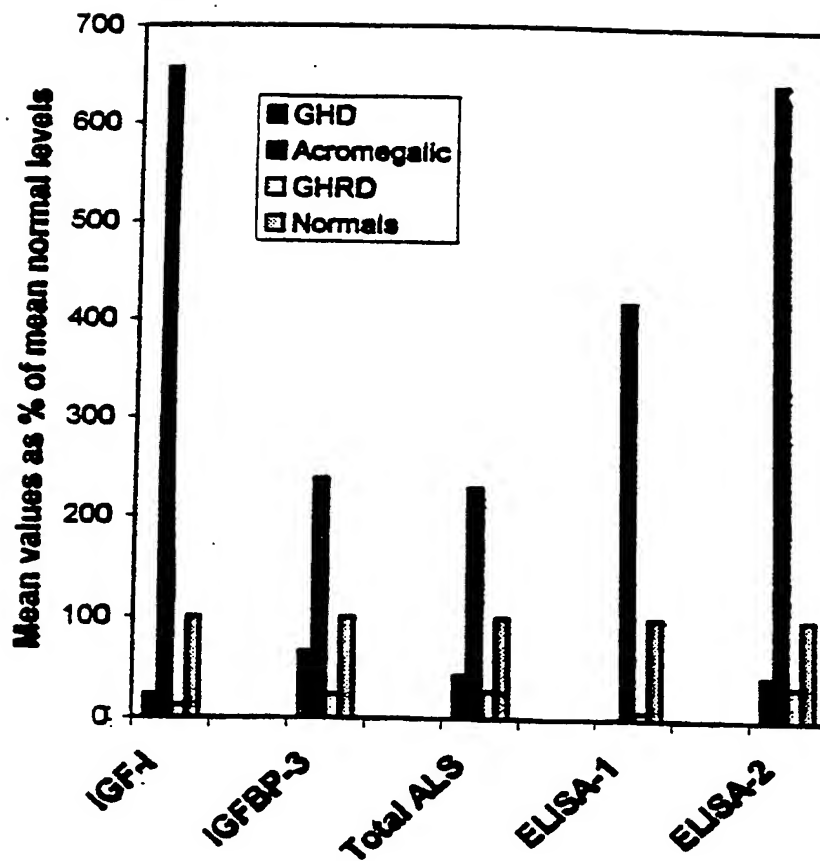
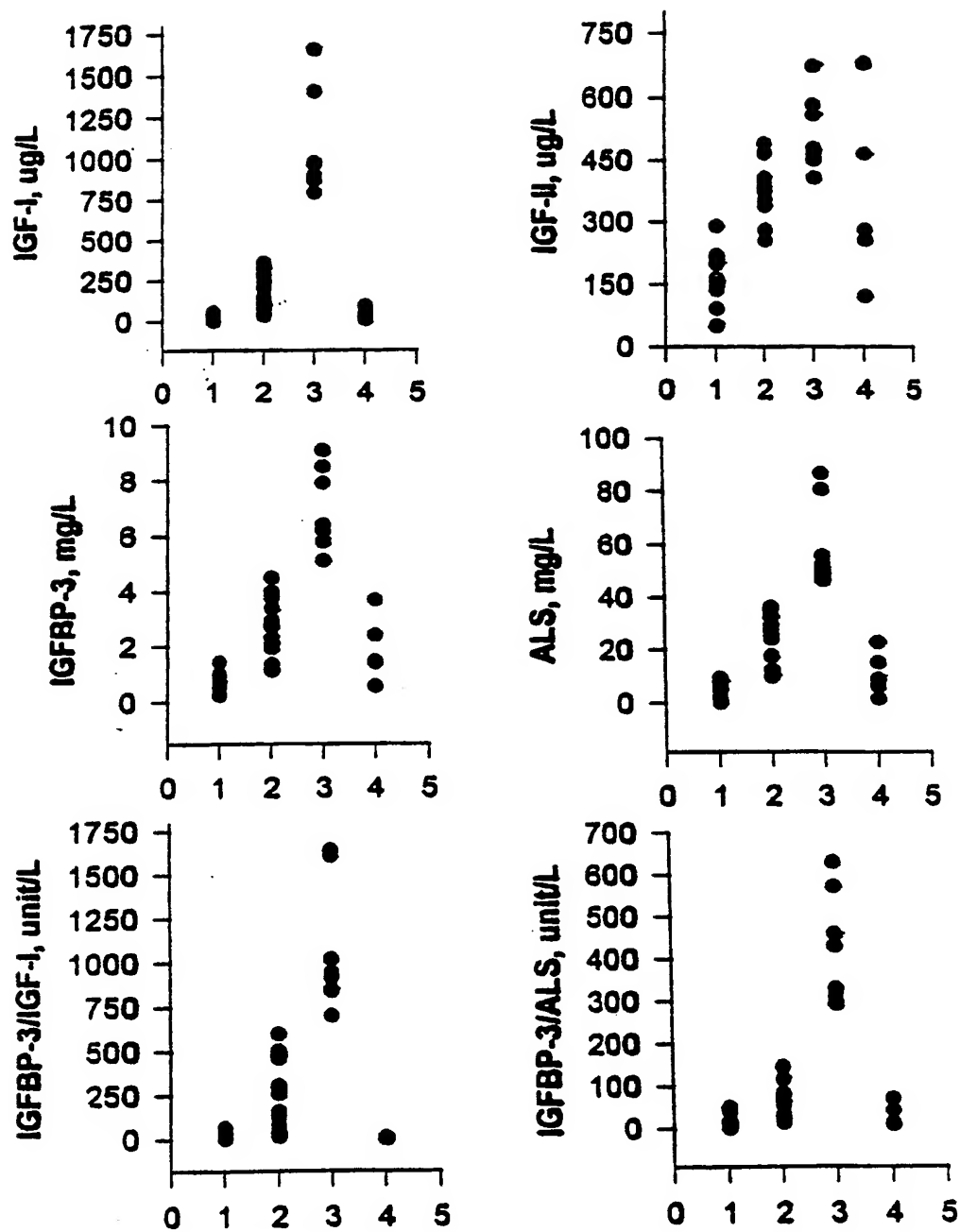


Figure 12



1=GHRD; 2=Normals; 3=Acromegalic; 4=GHD

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05210

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/543, 33/74

US CL :435/7.94, 7.5, 7.8, 334, 335, 336, 337; 436/518, 531

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS

search terms: IGFBP, IGF(w)BP, elisa, sandwich, two(w)site, insulin like growth factor binding protein

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	KHOSRAVI et al. Noncompetitive ELISA for Human Serum Insulin-Like Growth Factor-1. Clinical Chemistry. 1996, Vol. 42, No. 8, pages 1147-1154, see especially page 1149, column 1.	1-2, 6-8, 11, 14-16 ----- 1-21
X	RUTANEN et al. Measurement of Insulin-Like Growth Factor Binding Protein-1 in Cervical/Vaginal Secretions: Comparison with the ROM-Check Membrane Immunoassay in the Diagnosis of Ruptured Fetal Membranes. Clinica Chimica Acta. 31 January 1993, Vol. 214, No. 1, pages 73-81, see especially page 75.	1-2, 6-8, 11, 14-16
A	OY MEDIX BIOCHEMICA AB. Monoclonal Antibodies. Kauniainen: Oy Medix Biochemica AB, 1994, pages 15-16.	1-16



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

18 JUNE 1999

Date of mailing of the international search report

08 JUL 1999

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INTERNATIONAL SEARCH REPORT

 International application No.
PCT/US99/05210

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VORWERK et al. Synthesis of IGFBP-3 Fragments in a Baculovirus System and Characterization of Monoclonal Anti-IGFBP-3 Antibodies. Journal of Clinical Endocrinology and Metabolism. 1997, Vol. 82, No. 7, pages 2368-2370, see entire document.	1-21
Y	CAMPBELL, A.M. Monoclonal Antibody and Immunosensor Technology. Amsterdam: Elsevier, 1991, pages 2-6 and 42-45, see especially pages 3-6 and 45.	1-21
Y	HELLSTROM et al. In: Monoclonal Antibodies for Cancer Detection and Therapy. Edited by R.W. Baldwin et al. London: Academic Press, 1985, page 20, see entire document.	1-21
Y	STRASBURGER et al. Comparison of Five Commercial Immunoassay Kits for IGFBP3 Measurement. Growth Regulation. February 1994, Vol. 4, Supplement 1, page 138, Abstract No. II349, see entire document.	14-21
Y	DE BOER et al. Monitoring of Growth Hormone Replacement Therapy in Adults, Based on Measurement of Serum Markers. Journal of Clinical Endocrinology and Metabolism. 1996, Vol. 81, No. 4, pages 1371-1377, see entire document.	14-21
Y	TILLMAN et al. Biochemical Tests in the Diagnosis of Childhood Growth Hormone Deficiency. Journal of Clinical Endocrinology and Metabolism. 1997, Vol. 82, No. 2, pages 531-535, see entire document.	14-21
Y	MANES et al. Functional Epitope Mapping of Insulin-Like Growth Factor I (IGF-I) by Anti-IGF-I Monoclonal Antibodies. Endocrinology. 1997, Vol. 138, No. 3, pages 905-915, see especially pages 906-907.	1-2, 6-7, 10-11
Y	US 4,271,140 A (BUNTING) 02 June 1981, see entire document.	1-2, 6-7, 10-11
A	BARRECA et al. Effect of the Acid-Labile Subunit on the Binding of Insulin-Like Growth Factor (IGF)-Binding Protein-3 to [¹²⁵ I]IGF-I. Journal of Clinical Endocrinology and Metabolism. 1995, Vol. 80, No. 4, pages 1318-1324.	1-21

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/05210

B. FIELDS SEARCHED

Minimum documentation searched

Classification System: U.S.

435/7.94, 7.5, 7.8, 70.21, 172.2, 334, 335, 336, 337, 975; 436/518, 531, 811; 530/387.7, 387.9, 388.22-388.25, 389.2, 389.3, 391.1, 391.3; 935/104, 110

Figure 1

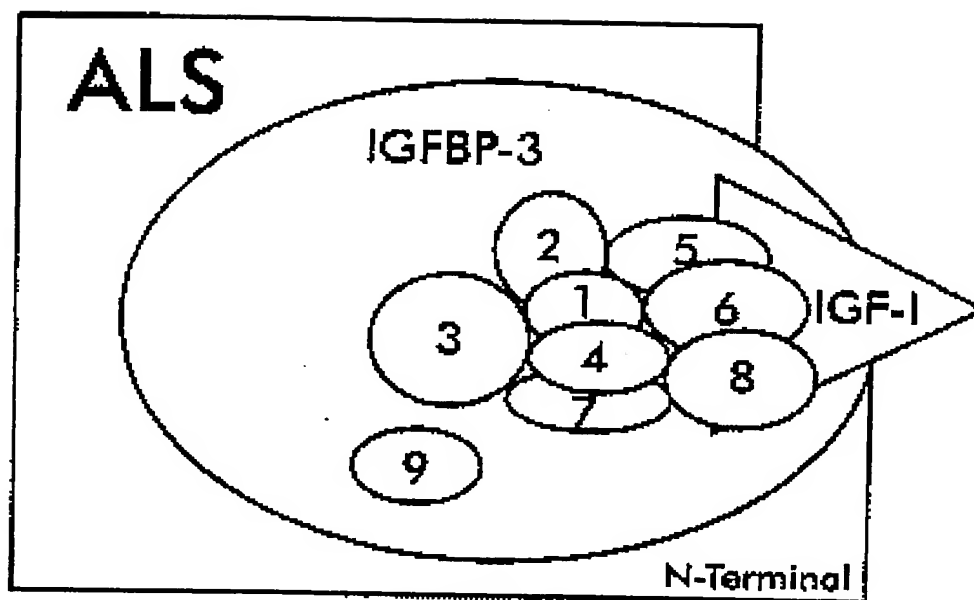


Figure 2

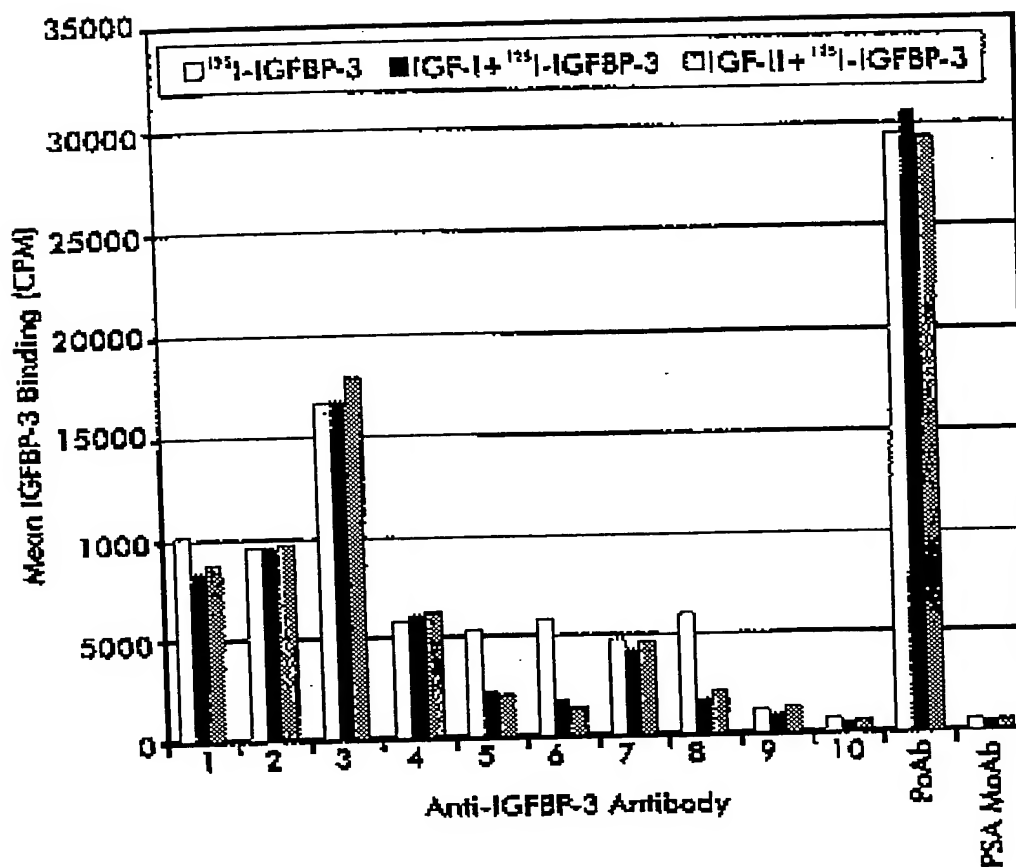


Figure 3a

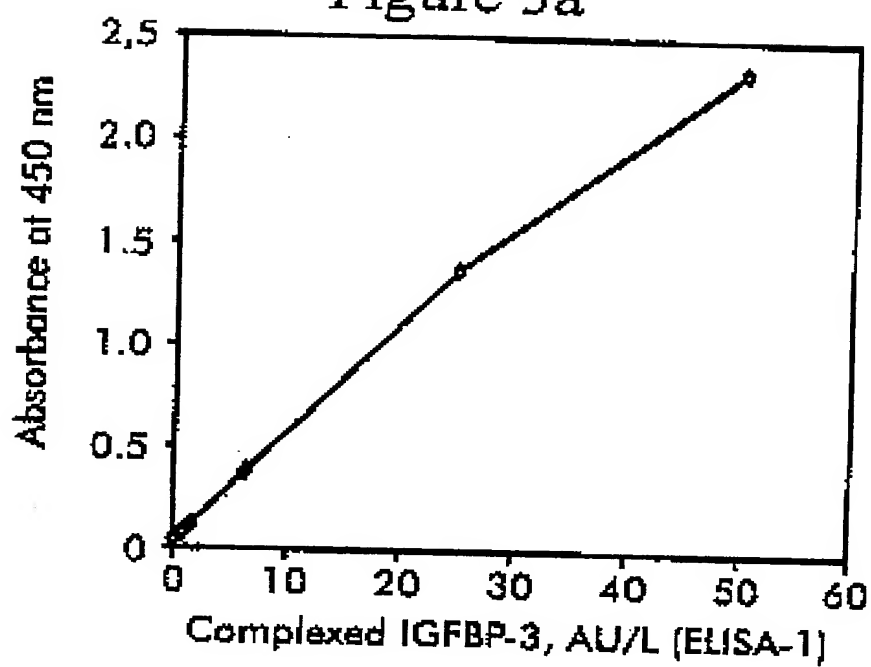


Figure 3b

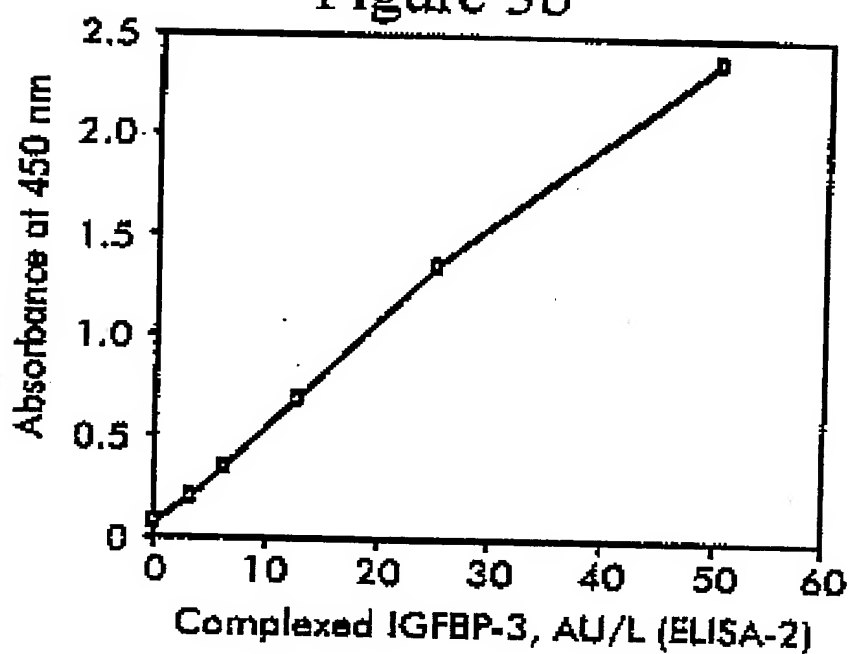


Figure 4a

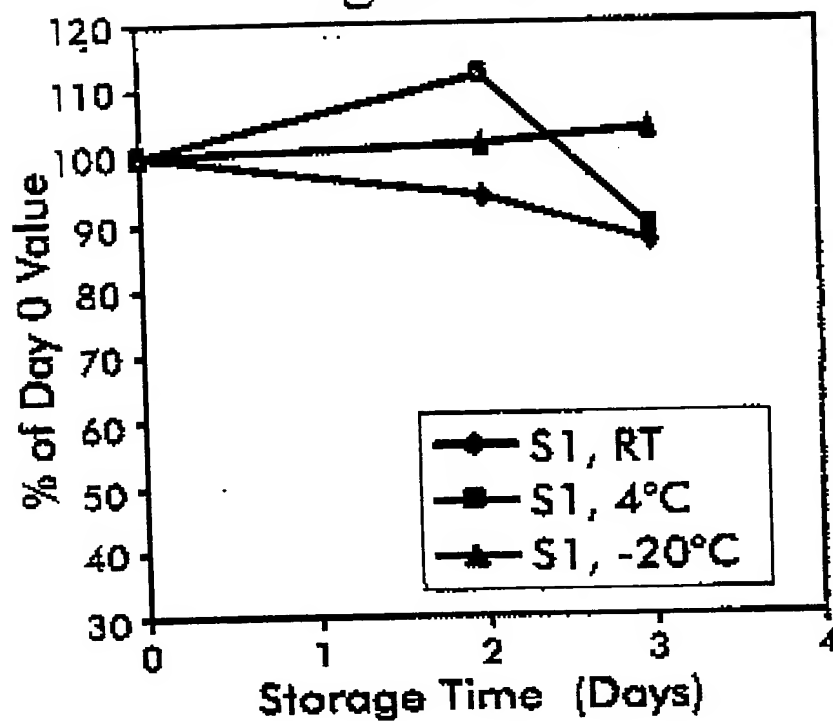


Figure 4b

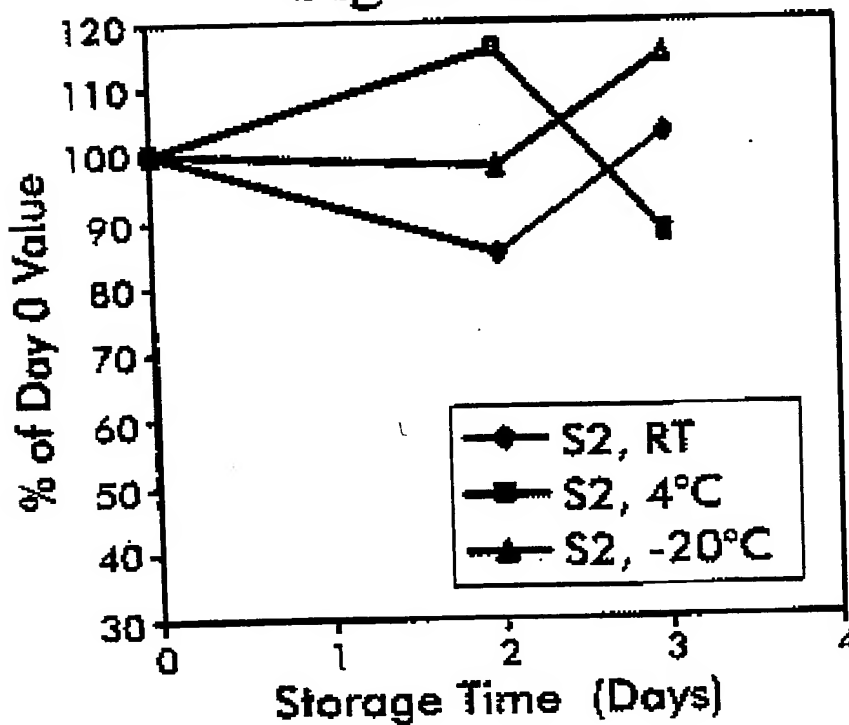


Figure 4c

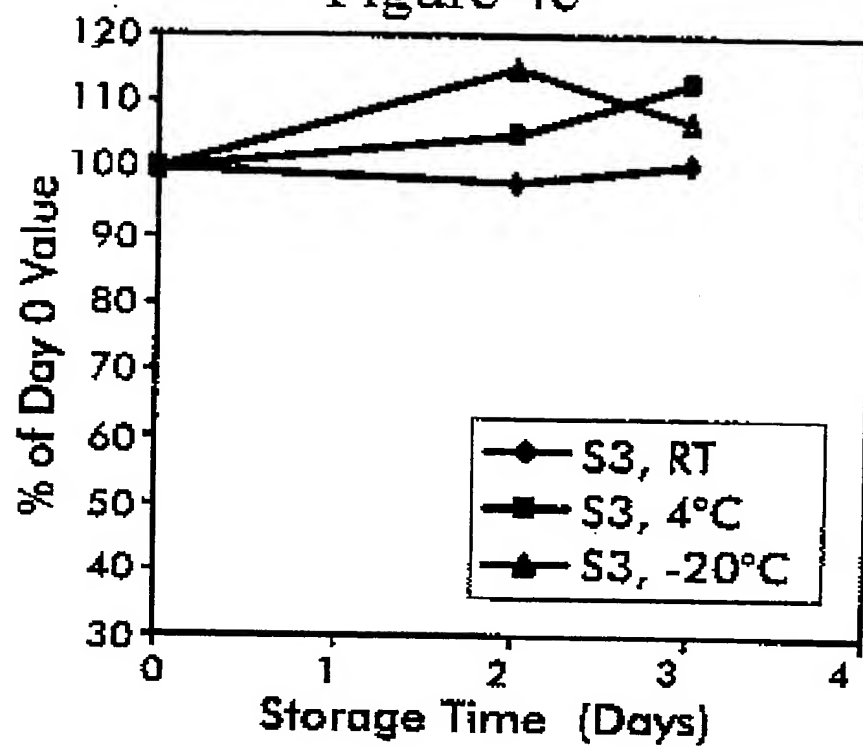


Figure 5a

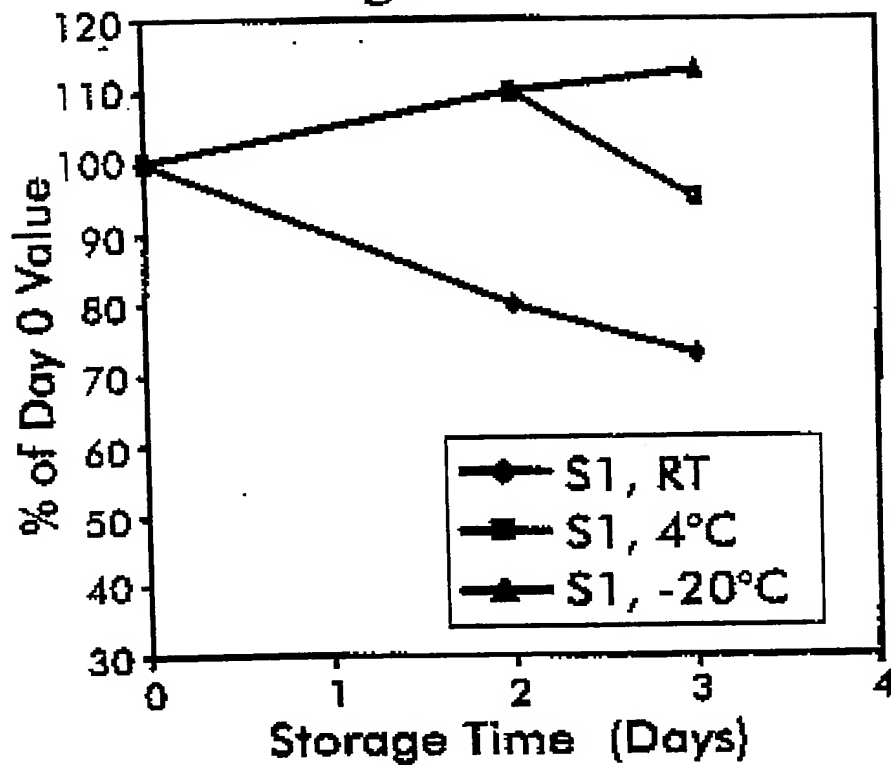


Figure 5b

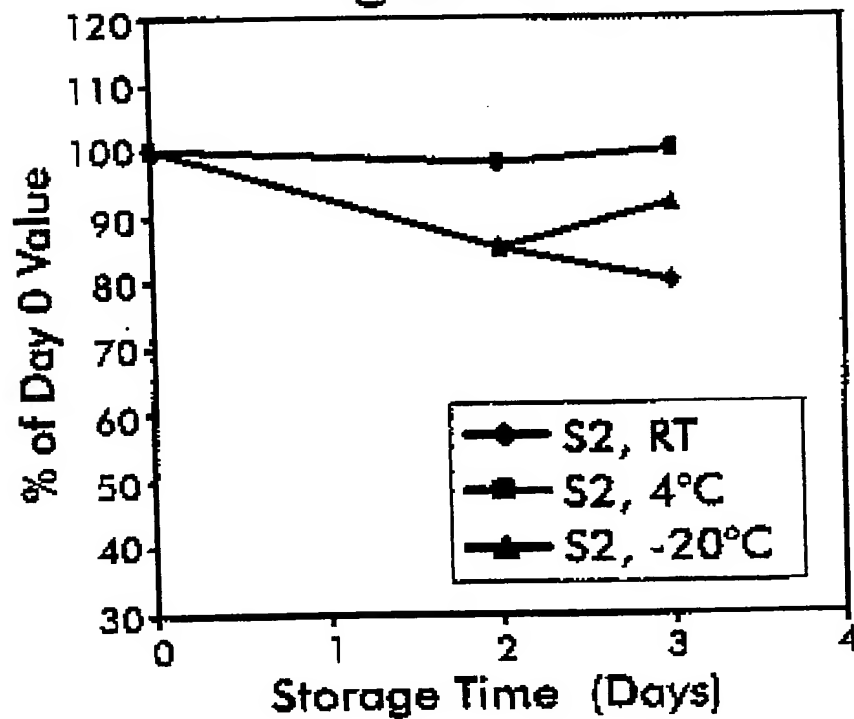


Figure 5c

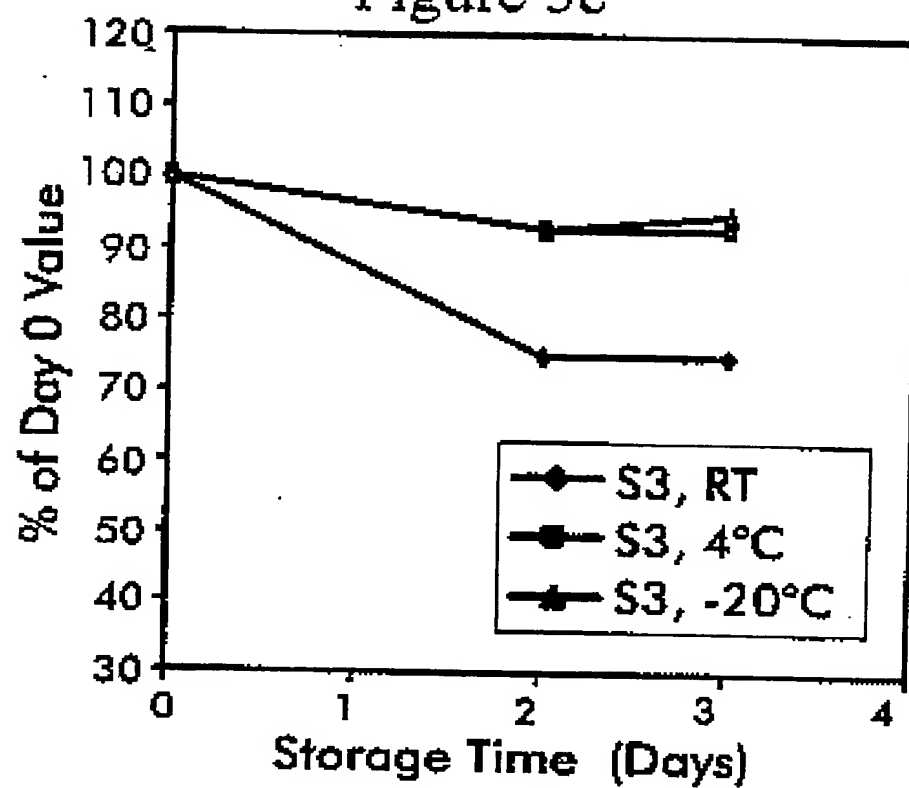


Figure 6a

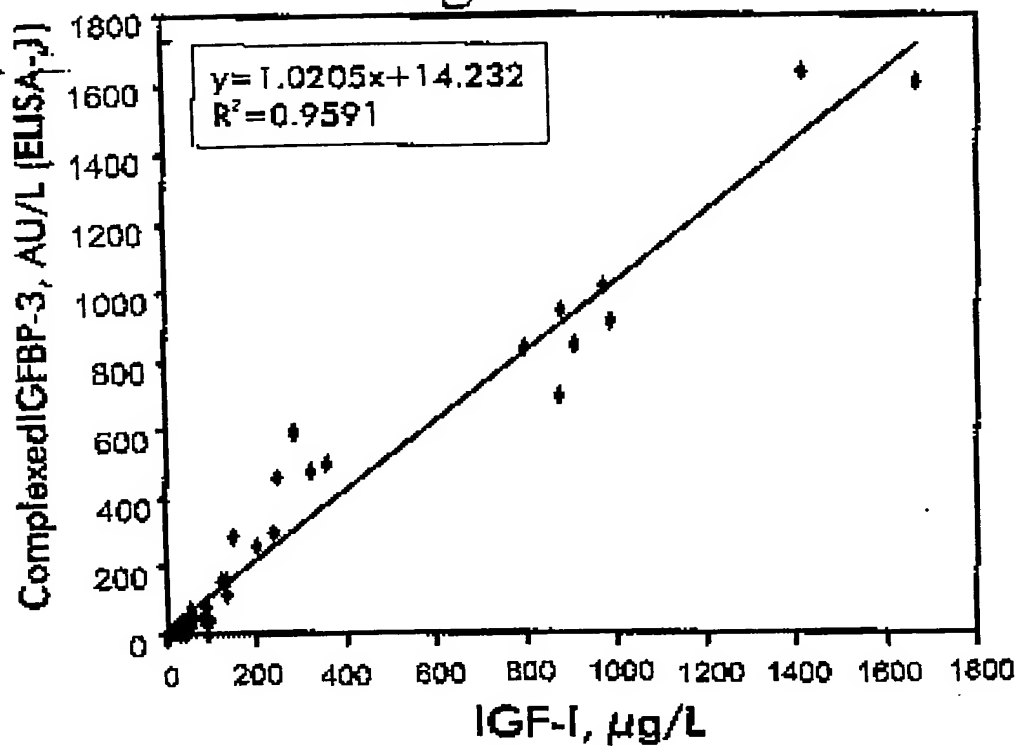


Figure 6b

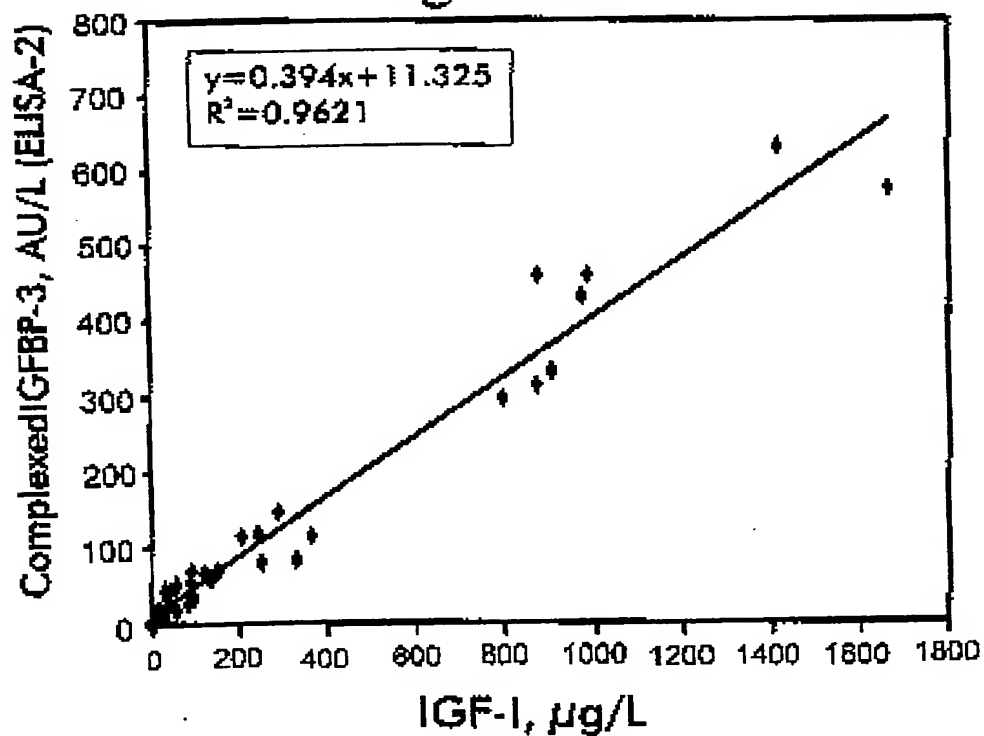


Figure 7a

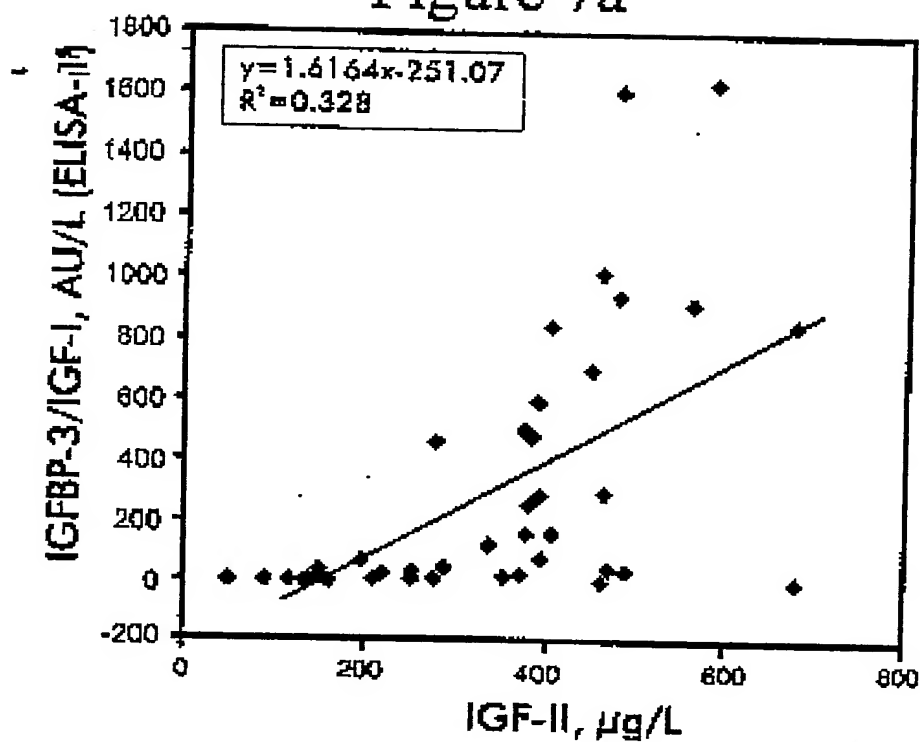


Figure 7b

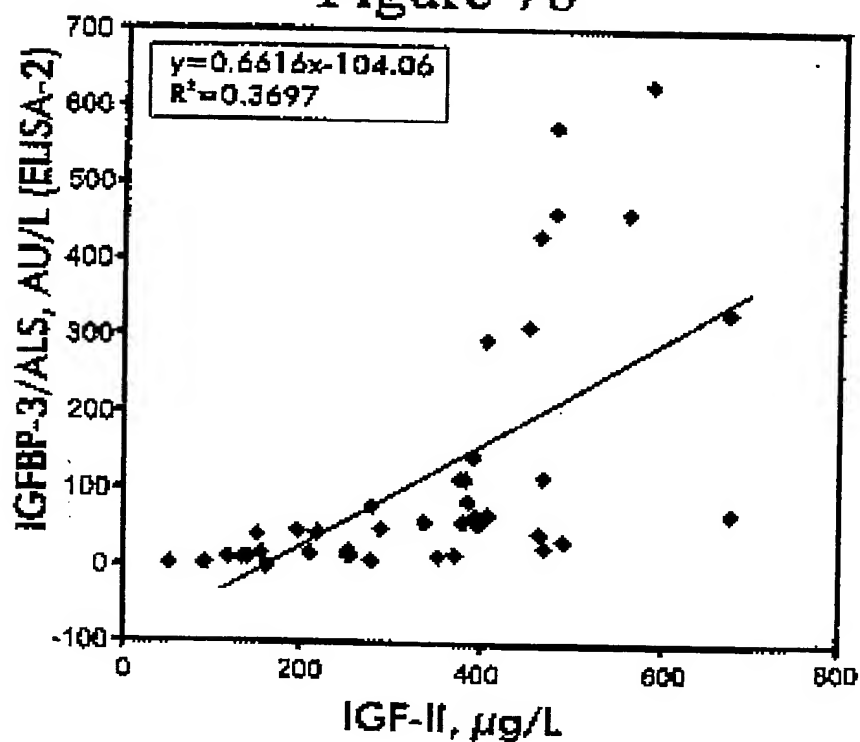


Figure 8a

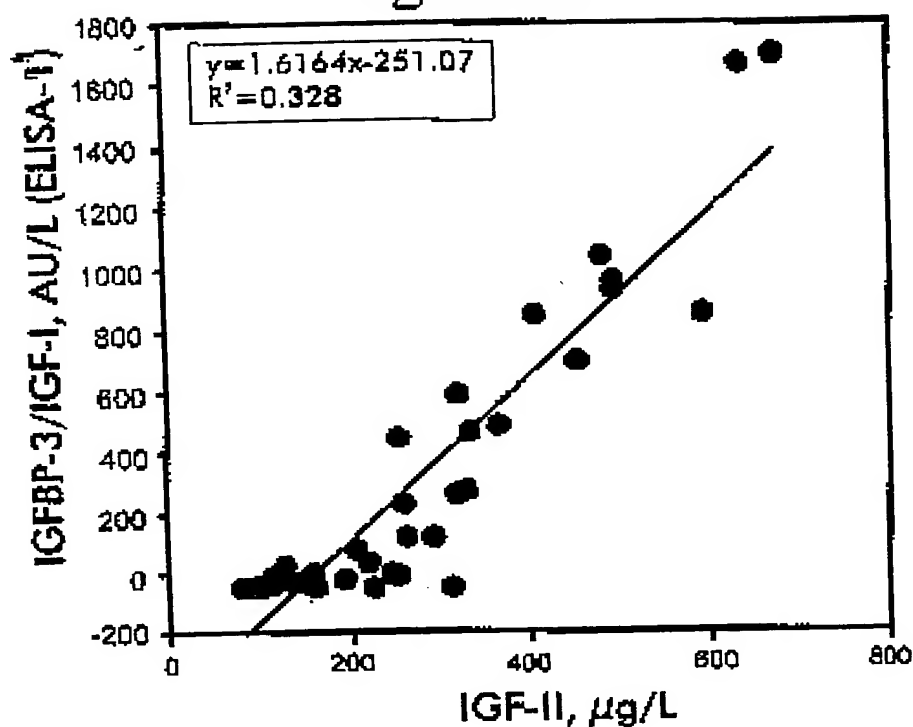


Figure 8b

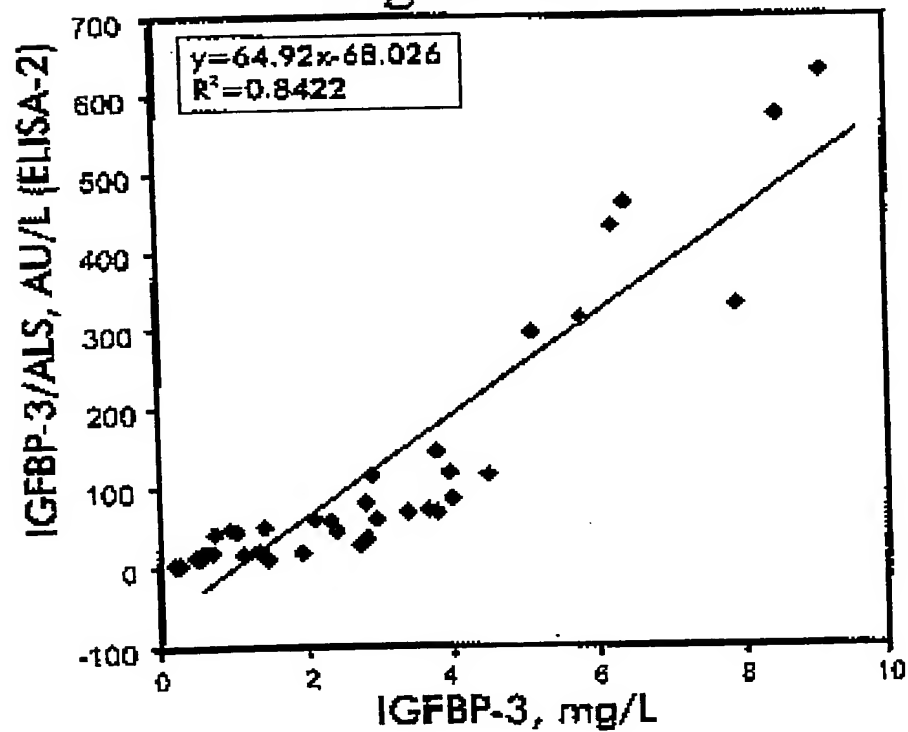


Figure 9a

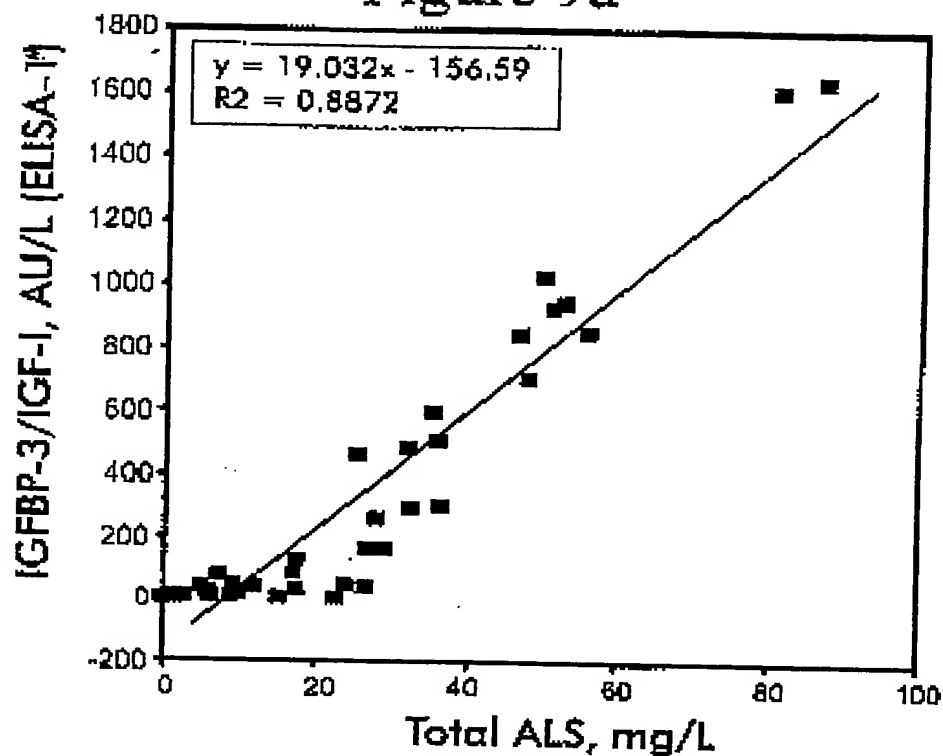


Figure 9b

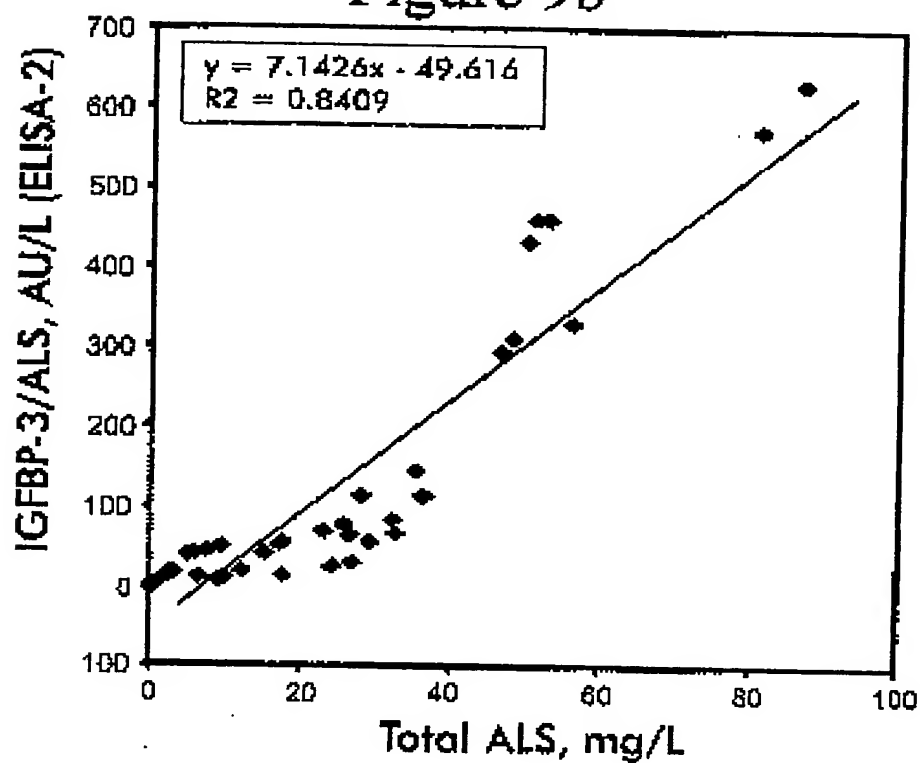


Figure 10

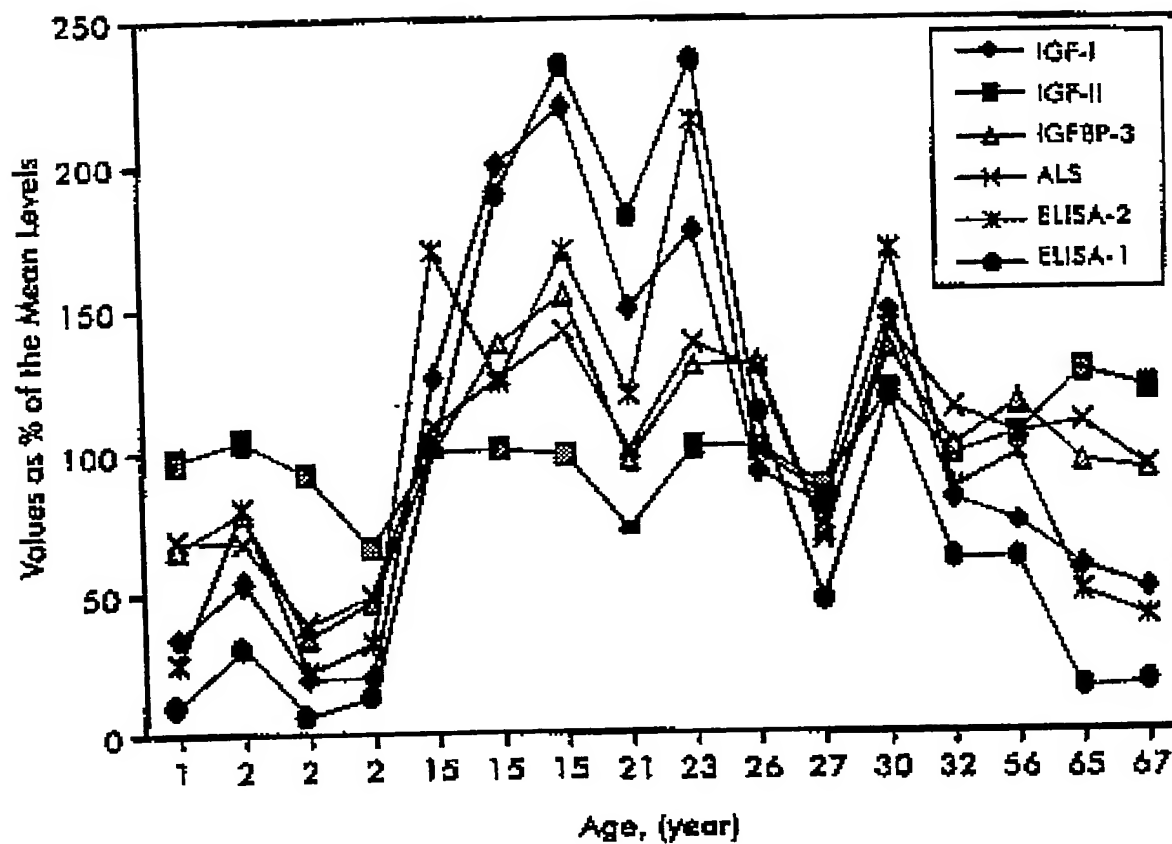


Figure 11

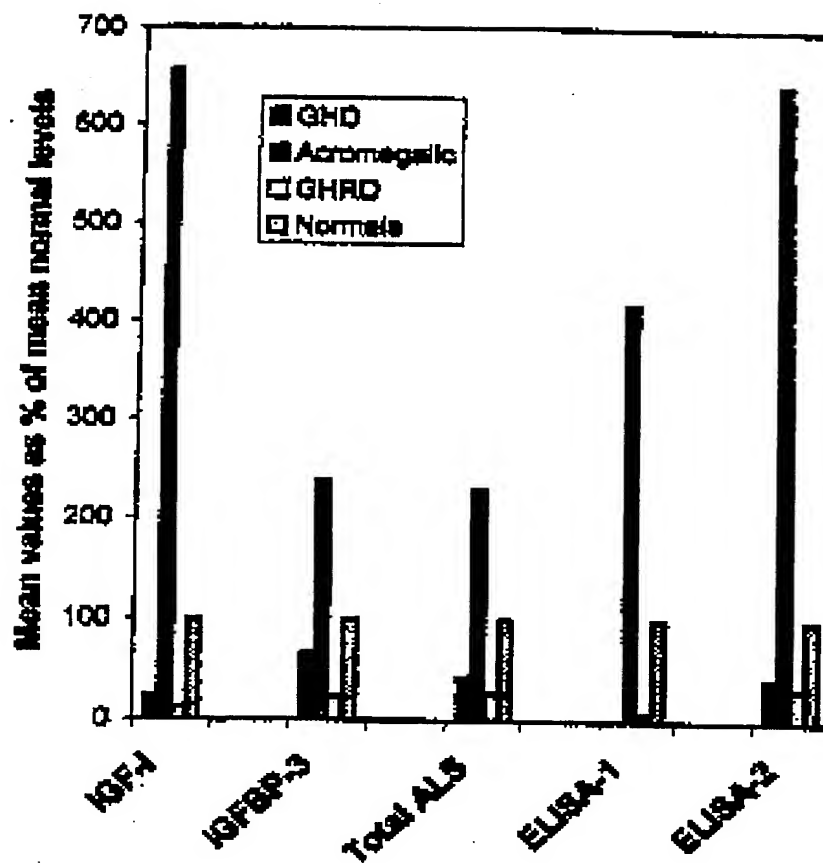
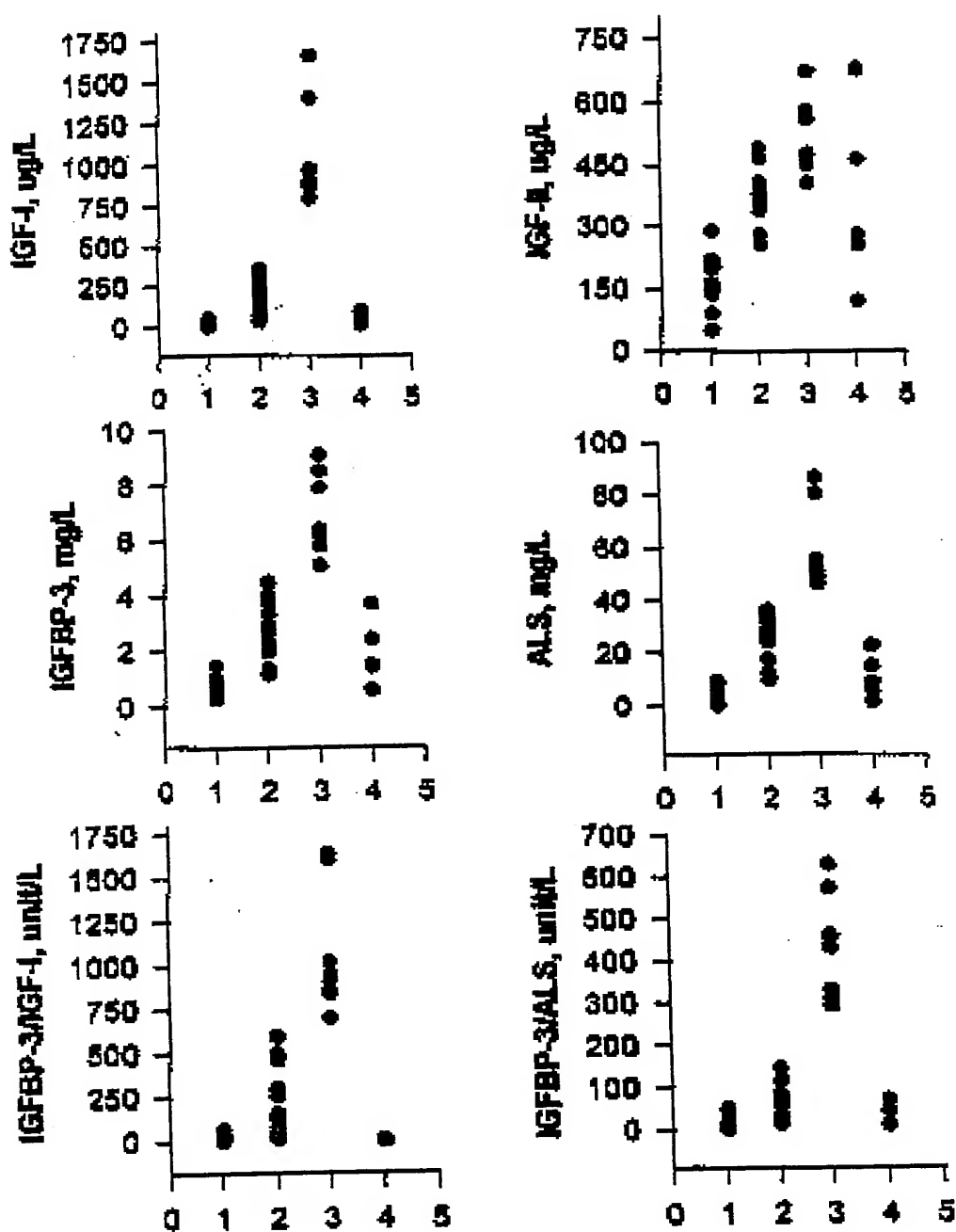


Figure 12



1=GHRD; 2=Normals; 3=Acromegalic; 4=GHD

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